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Diflubenzuron (Dimilin) : environmental effects and biochemical mode-of-action.

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DIFLUBENZURON (DIMILIN®): ENVIRONMENTAL EFFECTS AND BIOCHEMICAL MODE-OF-ACTION

A Dissertation Presented

by

PAULA J.S. MARTIN

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For Joe,

as always and forever

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ABSTRACT

DIFLUBENZURON (DIMILIN®): ENVIRONMENTAL EFFECTS AND BIOCHEMICAL MODE-OF-ACTION

SEPTEMBER 1993

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The effects of a single aerial application of diflubenzuron (Dimilin® over Warwick State Forest, MA, on nontarget aquatic macroinvertebrates of vernal pools and a stream was studied. Vernal pool populations which significantly decreased after the treatment were mosquito (Culicidae) pupae and *Cyclops*. No impact was demonstrated for the other abundant taxa in these vernal pools (mosquito larvae, water mites (Hydrarchanidae), tardigrades and springtails (Collembola)). The stream nontarget populations which significantly decreased after the treatment include 3 taxa of black flies (*Prosimulium mixtum/fuscum*, *Simulium vernum* & *S. vittatum*; Diptera: Simuliidae). No impact was demonstrated for the other abundant taxa (*Amphinemora*, *Leuctra*, *Ostrocerca* (Plecoptera); *Siphlonurus* (Ephemeroptera); *Rhyacophila*, *Ironoquia*, *Lepidostoma*, *Neophylax* (Trichoptera); Chironomidae (Diptera)) nor for a particular size class of these taxa. The timing of population growth and

development, and pesticide exposure by filter-feeding are the reasons used to explain these results.

A laboratory acute toxicity study of diflubenzuron with mosquito larvae (*Aedes aegypti;* Culicidae) under different water acidities was conducted. Low pH treatments (pH=4.5) resulted in 100 fold higher mortality of 4-day-old larvae (LC_{50}= 5 n*M*) compared with less acid water (pH=6.6; LC_{50}=500 n*M*). The synergist action of diflubenzuron with lowered pH has implications for prediction of nontarget impact in habitats exposed to acid rain.

A diflubenzuron biochemical mode-of-action study was conducted to determine if: (1) dolichol is present in the chitin-synthesizing plasma membrane of *Chironomus tentans* cell line, (2) dithiocarbanilates (i.e. nucleoside-transport inhibitors that affect membrane characteristics) inhibit chitin synthesis as does diflubenzuron in *C. tentans*, and, (3) diflubenzuron has a binding affinity for dolichol pyrophosphate N-acetylglucosamine (Dol-PP-GlcNAc). Plasma membrane was isolated from *C. tentans* by the method of Chaney & Jacobson (1983). No dolichol was found in this preparation, however, the preparation was never characterized as plasma membrane. Low cell culture growth prevented further study. Dol-PP-GlcNAc was produced from rat liver enzymes, however, diflubenzuron quantification limits (HPLC, ELISA) were too low to conduct a binding affinity study.

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LITERATURE REVIEW

Pesticides have been is use since ancient times to control insect pests. These first generation pesticides were derived from plants; the tobacco plant provided nicotine that was sprayed over plants to control insects in the 1700s (Matsumura 1989). Born in an era of subsistence agriculture and poor extraction techniques, botanical pesticides were expensive, and their effectiveness was variable. The next development in chemical pest control was the use of inorganic compounds (e.g., arsenicals) and petroleum products to kill insects. These second generation pesticides weren't very effective and they were very persistent. Their persistence caused toxicity problems to nontarget organisms. However, their inorganic nature meant they were not bioavailable and they did not move within the environment, making the nontarget problem locally limited.

Extremely effective, third generation pesticides were developed by organic chemists and were in use by the 1940s. These are the synthetic organic compounds. Chlorinated hydrocarbons were developed first, then organophosphates, carbamates and pyrethroids. All these compounds are nerve poisons; they are potentially toxic to any organism with a nervous system. They are also hydrophobic; they partition into (i.e. associates

with) organic matter (e.g., soil, leaves, sediment or fatty tissue). Chlorinated hydrocarbons are extremely persistent, with a half-life of years in the natural environment. In general, OPs are also persistent, but carbamates and pyrethroids are less so (Matsumura 1989).

The combination of persistence and hydrophobicity led to problems of bioaccumulation and biomagnification. Therefore, nontarget effects occurred on a global level, rather than the local effects with second generation materials.

Fourth generation pesticides are much more specific to the insect pest. Their use reduces the nontarget, ecologically disruptive aspects of synthetic organic compounds by targeting specific pests. One of these fourth generation pesticides is diflubenzuron.

Diflubenzuron

Diflubenzuron (Dimilin®, N-[{(4-chlorophenyl)amino}carbonyl]-2,6-difluorobenzamide, CAS #35367-38-5) is a chitin synthesis inhibitor (Post & Vincent 1973). It is toxic to insects and other arthropods that use chitin as a structural component of their cuticle. The other major taxa that uses chitin, the fungi, are not effected by diflubenzuron. Mammals, birds, and other vertebrates are not killed by diflubenzuron at dosages 10,000 times higher than

dosages used for arthropods (Miller et al. 1975; Julin & Saunders 1978; Gartrell 1981; Hudson et al. 1984). The specific action of diflubenzuron on arthropods classifies it as a fourth generation pesticide. This specific toxicity reduces the risk of environmental damage, compared with broad spectrum, third generation pesticides.

Diflubenzuron is also known as an insect growth regulator since it kills larvae when they molt. Adult insects are not killed by diflubenzuron though their reproductive abilities may be affected. Egg production and viability may be reduced after diflubenzuron exposure (Zaki & Gesraha 1987; Mittal & Kohli 1988).

Diflubenzuron was first approved for use in 1976. It is now registered for use against forest, cotton, soybean and mushroom insect pests (Eisler 1992). It is also registered for mosquito control in temporary pasture pools in California and Florida. Diflubenzuron is a restricted use pesticide due to its toxicity to nontarget aquatic macroinvertebrates (U.S. EPA 1985).

Effect on Nontarget Aquatic Organisms

Acute Laboratory Tests

Eisler (1992) reviewed diflubenzuron effects on nontarget aquatic macroinvertebrates in acute laboratory tests. Crustaceans are the most sensitive; adverse effects appear between 0.062 - 2.0 µg/L. Early

crustacean life stages are most vulnerable. Mosquito (Culicidae) larvae are the most sensitive aquatic insects. Ho et al. (1987) reported an LC_{30} (i.e. the lethal concentration at which 30% of the population dies) of 0.25 ng/L for second instar *Aedes albopictus*. Other immature aquatic insects have low survival and emergence rates at dosages of 0.1 - 1.9 µg/L. Adult aquatic beetles have no diflubenzuron-induced mortality at 250 µg/L. Fish and algae will bioaccumulate diflubenzuron though no adverse growth or mortality effects occur at dosages <1000 µg/L (Eisler 1992).

Field Tests

The taxa shown to be sensitive to diflubenzuron in acute toxicity tests are also sensitive in field situations. Field tests of diflubenzuron effects on nontarget aquatic organisms differ from acute toxicity studies in that populations recover quickly from diflubenzuron treatment, primarily because of rapid recolonization. Additionally, fish do not bioaccumulate diflubenzuron in the field (Colwell & Schaefer 1980), indicating that bioavailability and exposure routes are important in determining diflubenzuron mortality. Fish diets may be altered by diflubenzuron but their growth rates do not change (Colwell & Schaefer 1980).

Single Application. Studies of single diflubenzuron applications in the field have shown only short-term population effects that are reversed by recolonization. In a small stream, Mohsen & Mulla (1982) applied diflubenzuron at 0.1 µg/L for 15 min and found a moderate decline in black fly (*Simulium*) larvae, mayfly (*Baetis*) nymphs and net-spinning caddisfly (*Hydropsyche*) larvae.

In a large river, Satake & Yasuno (1987) applied diflubenzuron at 1.25 µg/L for 1 h and found most of the aquatic invertebrate populations were reduced or eliminated 1 week later. Recovery occurred quickly (by 3-4 weeks) for populations of fast recolonizers (e.g., *Similium, Baetis*). In an outdoor experimental stream, Yasuno & Satake (1990) applied 1000 times higher doses of diflubenzuron (1 mg/L or 10 mg/L) for 30 min. Midge (Chironomidae) larvae suffered high mortality and emerging populations of mayflies and caddisflies were reduced.

Swift et al. (1988) applied diflubenzuron to leaf packs (10 mg AI (i.e. active ingredient)/m^2) in a stream and found no short-term effect of treatment when recolonization was possible. They did find mortality to shredders (i.e. insects that feed on leaves or other coarse organic matter) fed on diflubenzuron-treated leaves in the laboratory.

In lentic (i.e. standing) water habitat, the effect of a single diflubenzuron application is comparable to the stream effects: short-term population reductions that are reversed by recolonization. Ali & Mulla (1978) found the nontarget impact on lake macroinvertebrates was more severe and longer lasting when most or all the habitat was treated as compared to partial treatment in semi-isolated lake fingers.

Ali & Lord (1980) applied diflubenzuron at 28 and 56 g AI/ha to a pond for chironomid control and found reduced *Cyclops* spp., Collembola, *Chaoborus* spp. and *Baetis* spp.

Sundaram et al. (1991) used 70 g AI/ha over an Ontario forest and studied permanent ponds within the forest. They found *Caenis* (mayfly), *Celithemis* (dragonfly), and *Coenagrion* (damselfly) significantly reduced 21-34 d post-treatment. Zooplankton (cladocera and copepod) were reduced 3 d after treatment and remained suppressed for 2-3 mo. None of the other 6 taxa studied were affected by the diflubenzuron treatment. Apperson et al. (1978) applied diflubenzuron to farm ponds to a final concentration of 2.5 ng/L that resulted in inhibited *Chaoborus* (phantom midge) emergence and suppressed zooplankton populations. Cladocerans were more susceptible than copepods, and required longer recovery periods. Fish

switched prey following treatment but there was no alteration in their growth.

Miura et al. (1976) used diflubenzuron at 7.2 and 8.1 g AI/ha for *Culex tarsalis* control in the California Central Valley foothills. The treatment was relatively safe to other organisms though cladoceran populations were reduced but recovered within 3 weeks.

In summary, field populations of microcrustaceans (cladocerans and copepods), and mosquitoes were reduced by diflubenzuron at application rates as low as 7.2 g AI/ha. Black flies, mayflies and caddisflies were reduced with diflubenzuron applied at 0.1 µg/L or 28 g AI/ha. Adult aquatic insects, aquatic Hemiptera, algae and fish were not affected by diflubenzuron applications.

Multiple Applications. A few studies have determined the effect of multiple applications of diflubenzuron on nontarget aquatic populations. Farlow et al. (1977) made 6 applications of diflubenzuron at 28 mg AI/ha to a Louisiana coastal marsh over 18 months. Five populations were significantly reduced: *Trichocorixa louisianae* (a waterboatman) immatures and adults, *Buenoa* spp. (a backswimmer) immatures, *Berosus infuscatus* (a water scavenger beetle) immatures, damselfly (Coenagrionidae) nymphs and all age classes of *Hyalella azteca* (an amphipod).

Twenty six taxa of aquatic insects, crayfish and fish did not significantly decrease in the treated marsh.

Hansen & Garton (1982) compared single species toxicity tests with community-level effects. Single species tests predicted the concentrations of diflubenzuron that affect stream communities; the most-sensitive acute toxicity test species were up to an order of magnitude more sensitive than the observed community level effects.

In summary, taxa affected by diflubenzuron in acute studies have correlated well with taxa affected in the field, although the mortality level is less than predicted by laboratory tests. Aquatic habitats tested to date include farm ponds (Miura et al. 1976; Apperson et al. 1978), forested ponds (Sundaram et al. 1991), lakes (Ali & Mulla 1978), small streams (Mohsen & Mulla 1982), outdoor experimental streams (Yasuno & Satake 1990) and large rivers (Satake & Yasuno 1987).

Because diflubenzuron inhibits a biochemical pathway absent in vertebrates (i.e. chitin synthesis), it appears especially safe for use (Muzzarelli 1987). However, the biochemical mode-of-action of chitin synthesis inhibition is unknown.

Mode-of-Action

Research on the biochemical effects of diflubenzuron has led to a number of theories of diflubenzuron mode-of-action. *In situ* tests of diflubenzuron inhibition, using whole animals, organs, tissues or whole cells, have shown diflubenzuron to interfere with cuticle synthesis, i.e. deposition and incorporation of chitin precursors (reviewed by Hammock & Quistad 1981). The first cell-free system of chitin synthesis was simultaneously produced in two different laboratories (Cohen & Casida 1980; Mayer et al. 1980). In cell-free systems, chitin synthesis was not inhibited by diflubenzuron, though it was inhibited by Polyoxin D and Nikkomycin. Polyoxin D and Nikkomycin are competitive inhibitors of chitin synthase (UDP-2-acetoamido-2-deoxy-D-glucose:chitin 4β-acetoamidodeoxy-D-glucosyltransferase, E.C. 2.4.1.16) in fungi and cause accumulation of uridine diphosphate N-acetyl-D-glucosamine (UDP-GlcNAc). Turnbull & Howells (1983) used a cell-free system from *Lucilia cuprina* that did show diflubenzuron inhibition to chitin synthesis. However, their preparation was from a homogenate fraction that sedimented at 1000 *g*, and was therefore likely associated with large cell fragments. These results imply that diflubenzuron does not act directly on chitin synthase, as was assumed earlier (Sowa & Marks 1975; Marks et al. 1982), and that whole cells or

large cell fragments are necessary for the inhibition to occur. The following are theories of diflubenzuron biochemical mode-of-action. Appendix A lists all the abbreviations.

Ecdysone Inhibitor

Yu & Terriere (1975, 1977) found that ecdysone metabolism was inhibited by diflubenzuron. Redfern et al. 1982 showed that diflubenzuron reduced the ecdysone titre before adult ecdysis in *Oncopeltus fasciatus*, although it had no effect on the production of ecdysones for last instar nymphs. Hajjar & Casida (1979) showed that diflubenzuron did not alter *in vivo* metabolism of α- or ß-ecdysone for *Oncopeltus fasciatus*. These studies provide examples of the conflicting results of diflubenzuron interference with ecdysone.

Additionally, measurements of chitinase and phenol oxidase are conflicting, with reports of their increase following diflubenzuron treatment (Ishaaya & Casida 1974; Yu & Terriere 1975, 1977) or no change (Deul et al. 1978; Hegazy 1984).

Chitin Synthase Zymogen Inhibitor

Another theory, put forward by Leighton et al. (1981) and Marks et al. (1982), proposed that diflubenzuron could inhibit enzymatic activation of a

chitin synthase zymogen. Turnbull & Howells (1983) believed this to be the best explanation for their results. They used a cell-free system from *Lucilia cuprina* that did show inhibition of chitin synthesis by diflubenzuron; 25% inhibition occurred at diflubenzuron concentrations of 5-8 μ*M* and maximum inhibition (50%) occurred at 50 μ*M*. However, their preparation was from a homogenate fraction that sedimented at 1000 *g*, and was therefore likely associated with large cell fragments, rather than a true cell-free system. Turnbull & Howells (1983) argued that if most of the chitin synthase is initially present as an inactive form, and diflubenzuron inhibits the activation of the enzyme, then chitin synthesis inhibition would result from diflubenzuron treatment.

Marks et al. (1982) found that diflubenzuron acts as a serine protease inhibitor, with some preference for chymotrypsin-like proteases. They demonstrated that known specific chymotrypsin inhibitors did inhibit chitin synthesis. The most active of those tested, chymostatin, had an I_{50} of 2.3 x 10-7 *M*. Diflubenzuron has an I_{50} of 9.4 x 10-11 *M* (Muzzarelli 1987).

Arguments against this theory of diflubenzuron acting as a chymotrypsin (i.e. a protease) inhibitor include Grosscurt and Jongsma (1987). They found that diflubenzuron did not cause chymotrypsin inhibition *in vitro*, therefore concluded that diflubenzuron would be

unlikely to cause chymotrypsin inhibition *in vivo*. Additionally, diflubenzuron disruption of chitin synthesis occurs rapidly, within 15 min in *Pieris brassicae* (Deul et al. 1978). Fast inhibition of an enzyme-dependent biochemical process would be unlikely to occur if the inhibitor acted on an inactive form of the enzyme. Only if most of the enzyme was present in the inactive state, and there was constant enzyme turnover, would fast inhibition occur. Therefore, the theory that diflubenzuron acts as an inhibitor of the chitin synthase zymogen (by inhibiting a chymotrypsin-like protease) is not well-supported.

Microtubular System Disturbed by Diflubenzuron

Chitin synthesis, in cultured wing imaginal discs from *Plodia interpunctella*, is inhibited by colcemid and vinblastine, which disturb functioning of the microtubular system (Oberlander et al. 1983). The hypothesis that microtubules act as guides for the movement of vesicles from the Golgi complex to the cell surface at the start of the insect molt period was put forward by Locke (1969, 1976). It is possible that diflubenzuron interferes with the microtubular system (Grosscurt & Jongsma 1987), although no further evidence is available to support this supposition.

Physical Membrane Disruption

Mayer et al. (1984) found rapid (< 5 min), irreversible inhibition of nucleoside (uridine, adenosine and cytidine) uptake in Harding-Passey melanoma cells treated with diflubenzuron. Nucleoside uptake was inhibited by 30% after treatment with 25 μM diflubenzuron, and inhibition was not reversible after washing the cells. They suggested that diflubenzuron may affect membrane properties in the same manner as some dithiocarbanilates that have structures similar to diflubenzuron (Kessel & McElhinney 1978). Dithiocarbanilate inhibition of facilitated transport of nucleosides across leukemia L1210 cell membranes was correlated with an increase in cell surface hydrophobicity (Kessel & McElhinney 1978). Diflubenzuron may act to change membrane hydrophobicity, and therefore, affect GlcNAc transport across the membrane to the site of chitin synthesis.

Interferes with Dolichol

Chitin synthesis in fungi is inhibited by Polyoxin D (competitive inhibitor of chitin synthase in fungi, causes accumulation of UDP-GlcNAc; insecticidal activity only after injection) and Nikkomycin (acts like Polyoxin D) via inhibition of the GlcNAc-polymerizing chitin synthase. Diflubenzuron shows no effect on fungi (Muzzarelli 1986) nor does Tunicamycin,

an inhibitor of N-acetylglucosaminephosphotransferase (UDP-N-acetylglucosamine:dolichyl-phosphate N-acetylglucosaminephophotransferase, E.C. 2.7.8.15; Cabib 1981). Chitin synthesis in insects is inhibited by diflubenzuron, Tunicamycin, Polyoxin D and Nikkomycin (the latter two inhibit *in vitro*, cell-free preparations). Chitin synthase is believed to be present on the extracellular side of plasma membranes (Mothes & Seitz 1981); there is no evidence that it is a transmembrane enzyme. Therefore, UDP-GlcNAc must cross from intracellular cytoplasm across the intracellular side of the plasma membrane to be available to the extracellular, membrane-bound chitin synthase. Phosphorylated compounds cannot passively penetrate cell membranes so there must be a mechanism of UDP-GlcNAc transport. Dolichol may play a role in this transport (Grosscurt & Jongsma 1987).

Mitsui et al. (1984, 1985) proposed that diflubenzuron interferes with UDP-GlcNAc transfer. Midguts of cabbage armyworm, *Mamestra brassicae* L. were incubated with [^{14}C]-GlcNAc. [^{14}C]-UDP-GlcNAc was produced in the presence or absence of diflubenzuron. However, transport of [^{14}C]-UDP-GlcNAc across the microvilli of the gut was inhibited by diflubenzuron, and [^{14}C]-UDP-GlcNAc accumulated in the treated tissue. Midgut cells produce the chitinous peritrophic membrane in the lumen of the gut. When [^{14}C]-UDP-GlcNAc was

present with diflubenzuron in the lumen of the gut, chitin synthesis was not inhibited. However, in the presence of Polyoxin D, chitin synthesis was inhibited. These results imply that diflubenzuron inhibits UDP-GlcNAc transport across the plasma membrane to chitin synthase. Oberlander et al. (1991) did not find amino sugar uptake into *Plodia interpunctella* wing disc cells to be inhibited by diflubenzuron. Mayer et al. (1984) found that diflubenzuron inhibited the transport of nucleosides in melanoma cell culture.

To summarize, to date the most likely explanation of diflubenzuron mode-of-action includes membrane disruption or UDP-GlcNAc transport inhibition, possibly via Dol-P.

Acid Rain and Aquatic Macroinvertebrates

Aquatic insects are impacted by xenobiotics other than pesticides. Fossil fuel combustion and smelting of sulfide ores are a major source of acidic precipitation (Likens et al. 1979). Natural waters may be acidified, resulting in decreases in populations and species diversity (Hall et al. 1980; Hudson & Berrill 1986; Burton & Allan 1986). Causes of aquatic invertebrate mortality with low pH can be direct physiological effects or indirect trophic or pathogen effects (France & Graham 1985). Direct effects include ion uptake and regulation alterations (Hall et al.

1988), metal toxicity (Leivestad et al. 1976), shifts to protein metabolism (Correa et al. 1986) and aluminum accumulation on respiratory surfaces (Havas 1986). Freshwater aquatic insects must continuously take-up sodium, potassium, chloride and calcium to survive (Sutcliffe & Hildrew 1989). For many species this absorption occurs through the cuticle, either via specialized respiratory structures (i.e. gills), specialized osmoregulatory structures (i.e., chloride epithelial, anal papillae) or scattered chloride cells (Merritt & Cummins 1984). Ion uptake is dependent on external concentrations; concentrations of physiologically-important ions (e.g., sodium) are low in acid-impacted waters (Sutcliffe & Hildrew 1989), resulting in decreased ion uptake and, possibly, death.

Life stage characteristics have been implicated in an individual's response to acid rain. Smaller organisms may be sensitive to a lower pH then when organisms are large (Havas 1981; Allard & Moreau 1987). Bell (1971) found insects near time of emergence were especially sensitive to lowered pH; molting arthropods are also especially sensitive (Sutcliffe & Hildrew 1989).

The response of fish to acidic conditions has been well-studied compared to the invertebrates. In general, aquatic invertebrates are more tolerant to elevated acidity and metals than many fish (Hall et al.

1988). The interactive effects on aquatic invertebrates of acid rain and xenobiotics such as diflubenzuron has not been reported.

CHAPTER 2

EFFECT OF DIFLUBENZURON ON NONTARGET AQUATIC MACROINVERTEBRATES

Introduction

Diflubenzuron (=Dimilin®) is an insecticide used on forests and field crops. It disrupts molting in arthropods by inhibiting chitin synthesis and weakening nascent cuticle; periods just before a molt are the most susceptible. Aquatic macroinvertebrates are highly susceptible to diflubenzuron, e.g., the LC_{50} for mysid shrimp is 2.06 ppb (U.S. EPA 1985). For this reason diflubenzuron is a restricted use pesticide.

Field studies of nontarget effects in freshwater include pond studies (Miura & Takahashi 1974, Ali & Lord 1980, Ali & Kok-Yokomi 1989, Sundaram et al. 1991), marsh studies (Farlow et al. 1977), and stream studies (Mohsen & Mulla 1982, Satake & Yasung 1987, Swift et al. 1988, Yasuno & Satake 1990, Sundaram et al. 1991). Short-term population reductions on some nontarget organisms were found to occur in each of these studies. Eisler (1992) reviewed the nontarget effects of diflubenzuron. Single field applications of diflubenzuron have shown only short-term population effects that are reversed through recolonization.

The purpose of this study was to determine the effects of a single, diflubenzuron aerial application on the nontarget invertebrate community in a stream and vernal pools.

Materials and Methods

Study Area and Treatment

The study area was the north-eastern portion of Warwick State Forest, Franklin County, Massachusetts (Fig. 1), an immature oak-hemlock forest (*Quercus* sp. and *Tsuga* sp.). Diflubenzuron (Dimilin® 25W) was applied, at 5.6 g AI/ha by a fixed wing airplane during the evening of May 18, 1989, to 300 forested acres for the control of oak-leaf tier larvae, *Croesia semipurpurana*. The control site was adjacent to the north border of the treated site. There was a single stream running through the control site into and through the treated site. Each site incorporated at least two vernal pools, extending from about 25-100 m^2. Sampling occurred from late April through mid-June.

Vernal Pool Sampling

Forested vernal pools, i.e., temporary, shallow, springtime pools with a substrate of decaying leaves, were sampled as long as they were constantly wet. Sampling frequency varied, with more frequent sampling near the date of the spray. Samples were taken on 4 different occasions before treatment.

Each site had two different, noncontiguous vernal pools, the larger of which was designated VP1 and the smaller VP2. A vernal pool sample consisted of a single dip of a long-handled, 0.335 ml dipper. VP1 had 20 samples and VP2 had 10 samples taken on each

sampling occasion. Occasionally, the number of samples at each pool was less than this. This was either due to dry-down of the pool, poor preservation, or samples lost due to leakage in the collection bag. VP2 at the treated site dried up following the first sampling day after treatment, therefore, it was not used for treatment effect analysis. Samples were placed in heavy plastic bags and transported back to the laboratory. They were kept cool until preserved; this occurred within 3 day of collection. Samples were preserved by first concentrating through a sieve (125 µm mesh; U.S. No. 120) then adding 70% ethanol. Taxa were identified to family level and counted. Mosquito larvae were categorized into 4 size classes, (1) < 0.20 mm, (2) 0.24-0.32 mm, (3) 0.36-0.44 mm, and (4) > 0.48 mm.

Stream Sampling

There was a single stream in the treated area; an upstream, unsprayed reach served as the control stream site. The stream was sampled in two different ways, with drift nets and with Surber samplers. Six drift nets were placed across two transects at each site and were left in place for 24 h intervals on each sampling occasion to collect all invertebrates carried by the water column. Six Surber samples, i.e. collections of invertebrates present on the stream bottom

(area: 22 cm x 30 cm), were randomly taken from each site, in areas upstream and downstream from the drift nets that were not previously sampled. There were 3 before treatment and 4 (drift) or 5 (Surber) after treatment sampling days. Samples were preserved in 70% ethanol and organisms sorted from organic debris under 20 x's magnification. Taxa were identified to lowest practical taxon and counted. Stream diversity was measured with Simpson's Diversity index, $1/C = (\sum(n-1))/(N(N-1))$; this is the inverse of Simpson's Index of Concentration, C (Routledge 1979; McElravy et al. 1989).

Insect growth regulators may act on a single age class while other age classes survive. For this reason, head capsule measurements were made on the abundant taxa with an ocular micrometer calibrated to 0.04 mm. Head capsule widths are an estimate of age of the individuals. For statistical analysis, the numbers of organisms were categorized into 5 or 6 arbitrary size categories. Simuliidae larvae were categorized into 5 size classes, (1) < 0.20 mm, (2) 0.24-0.32 mm, (3) 0.36-0.44 mm, (4) 0.48-0.56 mm and (5) 0.60-0.68 mm. Trichoptera and Plecoptera immatures were categorized into 6 size classes, (1) < 0.28 mm, (2) 0.32-0.48 mm, (3) 0.52-0.68 mm, (4) 0.72-0.88, (5) 0.92-1.08 mm, and (6) 1.12-1.28 mm.

Statistical Analyses

Data were subjected to log (x+1) transformation after determining heteroskedascity (Day & Quinn 1989) by the folded form F test (SAS Institute 1987). Planned comparisons of population abundances by site and time before treatment, and by site and time after treatment were conducted. If there was a significant site *x* time interaction term after-treatment due to population decrease in the treated site, or, if the population abundance significantly decreased after treatment in the treated site compared with the control site (PROC ANOVA, SAS Institute, 1987), then a treatment effect was declared (Green 1979). Populations with confounding before-treatment interactions or before-treatment significant differences were eliminated from analysis (PROC ANOVA, SAS Institute, 1987; Green 1979). This statistical analysis required equal sample sizes by site and by date. Missing values were replaced with the mean value for that date and site. The comparison of control VP2 (10 samples) with treated VP1 (20 samples) was conducted by randomly deleting 10 samples from the treated VP1 data set. If a site had data missing for an entire day, that day was deleted from statistical analyses for both sites. The overall experimentwise Type I error rate was kept to 0.05. Graphs represent untransformed means.

Treatment was not replicated therefore conclusions about the effect of the treatment are limited (Hurlbert 1984). However, large-scale treatments may have qualitatively different effects on ecosystems than small scale treatments, therefore it is important to document the effects of large scale applications even in the absence of replication (Carpenter 1990).

Results

Vernal Pools

These vernal pools did not have a diverse community of invertebrates. Immature mosquitoes, copepods and water mites were the most abundant organisms present; Collembola were occasionally quite abundant; Trichoptera, Chironomidae and Coleoptera were rare.

Figures 2-6 are graphical representations of the treatment and control site data, for the most abundant taxa, in each pool. With the exception of mosquito (Culicidae) pupae, no pretreatment organisms were completely lost from vernal pools after the diflubenzuron spray (Fig. 2-6).

The treated site VP1 was significantly different from the control VP1 for all taxa before the spray (Table 1). Treatment impact was documented for Culicidae only. Culicidae larvae (total and the 3 largest size classes) were more abundant in the

treated pool both before and after the treatment (Table 1, Fig. 2A, 3A & 4). However, Culicidae pupae were significantly less abundant after treatment in the treated site (none were found after May 24) while they remained in the control site though June 1 (Table 1, Fig. 2B & 3B).

The control VP2 was not significantly different from treated VP1 for Culicidae and *Cyclops* populations on the days before treatment (Table 2). Culicidae larval abundance (total or by each size class) was not significantly different between sites after the spray. Treated pool Culicidae pupal abundance is significantly less after treatment compared to control VP2 (Table 2, Fig. 2-4). *Cyclops* abundance in treated VP1 was significantly reduced with time compared to the control VP1 (Table 2, Fig. 5A). No discernible treatment effect was indicated for the other taxa due to significant site differences before the treatment.

Stream Results

Appendix B is a list of all the taxa collected at the treatment and control stream sites. Total diversity and total abundance of the control and treatment stream sites are presented in Fig. 7-8. There was no significant treatment effect on total diversity or total abundance.

Mean abundance over time and the mean abundance of head capsule widths over time for the stoneflies (Plecoptera) *Amphinemora* (Nemouridae), *Leuctra* type A and *Leuctra* type B (Leuctridae), and *Ostrocerca* (Nemouridae) that were commonly collected in either drift or Surber samples are presented in Fig. 9-18. The only mayfly (Ephemeroptera) that was common, and it was common only in the drift samples (Fig. 19), was *Siphlonurus* (Siphlonuridae). The caddisfly (Trichoptera) taxa that were commonly collected in either type of sampler are depicted in Fig. 20-25; taxa are *Ironoquia* (Limnephilidae), *Lepidostoma* (Lepidostomatidae), *Neophylax* (Limnephilidae) and *Rhyacophila* (Rhyacophilidae). Head capsule size analysis was conducted for *Rhyacophila* (Fig. 24-25). The true flies (Diptera) (Fig. 26-38) that were common in the stream samples include Ceratopogonidae larvae, Chironomidae larvae and pupae, and six black fly (Simuliidae) taxa: *Prosimulium magnum* complex, *P. mixtum/fuscum*, *P. rhizophorum*, *Simulium vernum* group, *S. vittatum* and *Stegopterna mutata* complex. Head capsule analysis was conducted for the black flies (Fig. 27-28, 30, 32, 35).

Treatment impact was demonstrated in drift and Surber samples for the black fly taxa: *Prosimulium mixtum/fuscum*, *Simulium vernum* group and *S. vittatum* (Tables 3 & 4). *Prosimulium mixtum/fuscum* species

complex was significantly reduced in Surber samples after treatment, with the rate of population decrease greater than seen in the control population. In drift samples, this taxon was significantly lower in the 4th size class, while other size classes did not have a significant decrease compared with the control. Surber samples of the *Simulium vernum* group in the treated reach were significantly reduced after treatment for all size classes expect size class 1 (Table 3). No impact effect could be concluded for size class 4 because this class was more abundant in the treated site before the treatment and less so after treatment. This could simply be the result of an earlier population in the treated reach (Table 3). Drift samples of the *S. vernum* group were significantly reduced (for total larvae and for size class 1) in the treated reach after treatment (Table 4). No conclusion could be drawn for the larger size classes due to significant interactions between sites by time before treatment (Table 4). *Simulium vittatum* were not present in drift samples until one day after the spray, at which time they had the same abundance as did the control reach (Table 4). The treated site had no *S. vittatum* after May 21 while the control site population abundance was increasing (Table 4).

Treatment impact could not be demonstrated for a larger size class if there was a significant, pre-

treatment difference in the younger size class since the younger size class is the recruitment source for the next size class (e.g., *Rhyacophila* size class 2, Table 3; *Stegopterna mutata* pupae, Table 4).

Discussion

Diflubenzuron aerial treatment had a detectable effect only on cyclopoid copepods and certain Nematocera (i.e. mosquitoes and black flies). These are all filter-feeding macroinvertebrates. Populations living in temporary habitats such as vernal pools vary with the natural cycling of their habitat (e.g., Fig. 5a). Therefore, separating population change caused by natural cycling of the vernal pool versus population mortality from a pesticide spray is somewhat problematic. This was made particularly difficult since the pesticide spray took place just 5 days before the dry down for one sampling pool. However, this illustrates normal catastrophic losses experienced by vernal pool organisms. Even with this constraint, a significant effect of the treatment was detected for Culicidae pupae and *Cyclops*.

Mosquitoes are known to be sensitive to diflubenzuron, especially during the final (4th) instar before the pupal molt (Mulla & Darwazeh 1975). This instar is feeding constantly as well as building the pupal cuticle, a cuticle that is thicker than the

larval cuticle (Clements 1992). Mosquito pupae were eliminated from treated vernal pools. *Cyclops* is another taxon known to be sensitive to diflubenzuron (Eisler 1992) and whose populations were reduced during this study.

There were six taxa of black flies in the treated stream; three were found to have a treatment effect using both types of sampling. Two of these (*S. vernum* group and *S. vittatum*) are multivoltine summer species. The other black flies are univoltine spring species. The timing of the spray in late spring, coincided with the normal population decrease for these univoltine, spring black flies. They were present as late instar larvae or pupae at the time of the spray. The only univoltine species to be impacted by diflubenzuron was *P. mixtum/fuscum*. Its most abundant size class at the time of the spray was the largest size class, and it was only this particular size class that was shown to be impacted by the treatment. Two of the impacted populations (*S. vittatum* and *S. vernum* group) were just beginning their growth for the year. At the time of the spray they were present as young instars only. Early instar *Simulium* are known to be more sensitive to diflubenzuron than late instars (Lacey & Mulla 1978). The timing of the spray in relation to life history parameter seems to be a critical factor in determining toxicity.

Filter-feeders may be more sensitive for two reasons. First is the partitioning characteristics of diflubenzuron. Diflubenzuron water solubility is only 0.3 ppm. Diflubenzuron adsorbs onto particulate, organic matter (Booth et al. 1987) making it more available to filter-feeding organisms. The second rationale is that the compound was applied as a wettable powder, with the active ingredient adsorbed onto clay particles. These particles could be ingested by filter-feeding organisms, resulting in their mortality.

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Table 1. Comparison of control vernal pool 1 to treated vernal pool 1.

Control VP1 vs Treated VP1	Before		After		Impact	Transformed Mean Abundance by Date									
Taxa	Site	Site by Date Inter-action	Site	Site by Date Inter-action		Before					After				
						5/5	5/12	5/13	5/18		5/21	5/23	5/28	5/31	6/1
Culicidae larvae, control	**	*	**	**	No	0.44	0.27	0.14	0.34		0.45	0.12	0.13	0.10	0.16
--treated						0.76	0.68	0.92	0.94		1.02	0.96	0.24	0.25	0.07
size class 1, control	NS	NS	0	0	No	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
--treated						0.02	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
size class 2, control	**	*	**	**	No	0.09	0.03	0.03	0.02		0.03	0.00	0.02	0.00	0.04
--treated						0.40	0.19	0.52	0.20		0.25	0.16	0.03	0.03	0.02
size class 3, control	**	NS	**	**	No	0.22	0.11	0.05	0.11		0.16	0.04	0.04	0.10	0.04
--treated						0.49	0.38	0.39	0.68		0.69	0.52	0.08	0.04	0.02
size class 4, control	**	NS	**	**	No	0.22	0.12	0.07	0.27		0.30	0.08	0.05	0.00	0.04
--treated						0.36	0.35	0.26	0.59		0.76	0.76	0.19	0.19	0.03
pupae, control	NS	*	**	NS	Yes[a]	0.00	0.08	0.05	0.33		0.62	0.62	0.20	0.13	0.16
--treated						0.00	0.02	0.00	0.56		0.42	0.17	0.00	0.00	0.00
Ergasilus, control	**	*	NS	NS	No	0.02	0.00	0.00	0.00		0.00	0.00	0.03	0.00	0.00
--treated						0.12	0.02	0.00	0.07		0.03	0.00	0.02	0.03	0.00
Cyclops, control	**	*	**	**	No	0.64	0.77	0.27	0.87		1.46	0.27	0.40	1.09	0.45
--treated						0.94	0.90	1.00	1.25		0.61	0.25	0.44	0.38	0.28
Acari, control	**	NS	**	NS	No	0.93	0.44	0.46	0.81		0.73	0.47	0.25	0.23	0.26
--treated						1.73	1.45	1.65	1.75		1.39	1.37	1.22	1.23	0.96
Collembola, control	**	NS	**	**	No	0.30	0.08	0.06	0.03		0.05	0.00	0.00	0.00	0.00
--treated						0.57	0.42	0.70	0.39		0.42	0.59	0.30	0.20	0.02
Tardigrada, control	**	*	*	NS	No	0.06	0.00	0.00	0.02		0.00	0.00	0.00	0.00	0.00
--treated						0.20	0.09	0.28	0.05		0.02	0.05	0.02	0.00	0.03

* Significant, $P \leq 0.05$; ** Significant, $P \leq 0.001$; [a] pupae absent after 5/24 in treated pool while present in control, and larval abundance significantly greater in treated pool; NS=not signficantly different.

Table 2. Comparison of control vernal pool 2 to treated vernal pool 1.

Control VP2 vs Treated VP1	Before		After		Impact	Transformed Mean Abundance by Date									
Taxa	Site	Site by Date Inter-action	Site	Site by Date Inter-action		Before					After				
						5/5	5/12	5/13	5/18		5/21	5/23	5/28	6/1	6/15
Culicidae larvae, control	NS	NS	NS	NS	No	1.24	0.81	0.59	0.81		0.90	0.82	0.31	0.12	0.00
--treated						0.84	0.86	0.85	0.90		0.86	0.91	0.26	0.06	0.00
size class 1, control	NS	NS	0	0	No	0.03	0.03	0.00	0.00		0.00	0.00	0.00	0.00	0.00
--treated						0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
size class 2, control	*	NS	NS	NS	No	0.98	0.74	0.41	0.24		0.10	0.13	0.03	0.00	0.00
--treated						0.48	0.28	0.29	0.20		0.25	0.18	0.00	0.00	0.00
size class 3, control	NS	NS	NS	NS	No	0.60	0.25	0.29	0.58		0.64	0.50	0.12	0.34	0.00
--treated						0.47	0.47	0.43	0.69		0.61	0.50	0.09	0.00	0.00
size class 4, control	*	NS	NS	NS	No	0.53	0.06	0.06	0.35		0.59	0.45	0.20	0.09	0.00
--treated						0.54	0.44	0.25	0.52		0.57	0.71	0.18	0.06	0.00
pupae, control	*	**	*	NS	Yes[a]	0.00	0.00	0.00	0.14		0.42	0.49	0.12	0.07	0.00
--treated						0.00	0.00	0.00	0.48		0.32	0.11	0.00	0.00	0.00
Ergasilus, control	*	NS	**	*	No	0.10	0.17	0.03	0.25		0.31	0.28	0.06	0.27	0.00
--treated						0.08	0.03	0.00	0.03		0.00	0.00	0.00	0.00	0.03
Cyclops, control	NS	NS	**	*	Yes[b]	0.97	0.63	0.76	1.05		0.83	0.95	0.37	0.86	0.03
--treated						0.90	0.77	1.13	1.18		0.54	0.34	0.45	0.34	0.09
Acari, control	**	NS	**	NS	No	0.69	0.34	0.45	0.46		0.14	0.33	0.29	0.09	0.12
--treated						1.63	1.38	1.80	1.68		1.33	1.27	1.20	1.04	0.73
Collembola, control	**	NS	**	*	No	0.07	0.03	0.03	0.16		0.06	0.06	0.06	0.00	0.06
--treated						0.45	0.45	0.55	0.31		0.41	0.55	0.30	0.20	0.05
Tardigrada, control	**	*	NS	NS	No	0.87	0.16	0.37	0.31		0.00	0.00	0.03	0.03	0.00
--treated						0.15	0.13	0.27	0.04		0.03	0.08	0.05	0.03	0.00

* Significant, $P \leq 0.05$; ** Significant, $P \leq 0.001$; [a] pupae absent from treated site after 5/24 while present in control and larval abundance not significantly less than control; [b] *Cyclops* abundance in treated pool significantly reduced with time compared with control while they were not signficantly different before the treatment; NS=not significantly different.

Table 3. Comparison of upstream control to downstream treated, Surber samples.

Control Surber vs Treated Surber	Before		After		Impact	Transformed Mean Abundance by Date							
						Before			After				
Taxa	Site	Site by Date Inter-action	Site	Site by Date Inter-action		5/7	5/11	5/17	5/20	5/22	5/27	6/1	6/15
Plecoptera													
Amphinemora, control	NS	NS	NS	*	N[a]	0.47	0.64	0.70	0.94	0.96	0.18	0.00	0.29
--treated						0.77	0.38	0.83	0.75	0.74	0.78	0.44	0.00
size class 2, control	NS	NS	NS	NS	No	0.37	0.48	0.28	0.38	0.28	0.00	0.00	0.00
--treated						0.72	0.30	0.28	0.20	0.23	0.00	0.00	0.00
size class 3, control	NS	NS	NS	NS	No	0.23	0.47	0.63	0.73	0.78	0.17	0.00	0.00
--treated						0.17	0.12	0.73	0.68	0.58	0.47	0.05	0.00
size class 4, control	0	0	NS	*	N[a]	0.00	0.00	0.00	0.27	0.47	0.05	0.00	0.08
--treated						0.00	0.00	0.00	0.25	0.28	0.57	0.30	0.00
size class 5, control	0	0	NS	**	N[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27
--treated						0.00	0.00	0.00	0.00	0.00	0.10	0.15	0.00
Leuctra Type A, control	NS	NS	**	**	N[a]	0.54	1.05	0.97	0.79	0.98	0.45	0.15	0.40
--treated						1.08	0.79	1.54	1.17	1.08	1.34	1.36	0.05
size class 1, control	NS	NS	**	**	N[a]	0.20	0.22	0.05	0.05	0.25	0.05	0.00	0.00
--treated						0.17	0.00	0.00	0.05	0.08	0.50	1.08	0.00
size class 2, control	NS	*	*	NS	N[a]	0.40	0.95	0.62	0.45	0.40	0.10	0.00	0.00
--treated						0.98	0.33	0.98	0.58	0.53	0.58	0.25	0.00
size class 3, control	*	NS	**	*	N[a]	0.10	0.55	0.63	0.57	0.83	0.37	0.15	0.30
--treated						0.48	0.68	1.38	1.03	0.97	1.18	0.88	0.05
size class 4, control	NS	NS	NS	*	N[a]	0.05	0.05	0.15	0.15	0.47	0.05	0.00	0.20
--treated						0.00	0.07	0.12	0.00	0.12	0.23	0.27	0.00
size class 5, control	NS	NS	NS	NS	No	0.00	0.00	0.13	0.05	0.08	0.00	0.00	0.00
--treated						0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00
Leuctra Type B, control	NS	NS	NS	NS	No	0.00	0.00	0.00	0.00	0.15	0.00	0.10	0.21
--treated						0.00	0.06	0.06	0.00	0.00	0.15	0.23	0.00

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Table 3. Continued.

Ostrocerca, control	**	*	**	*	No[c]	1.41	1.77	1.79		1.66	1.82	1.11	0.44	0.05
--treated						1.33	0.95	1.18		1.36	1.16	0.62	0.41	0.00
size class 2, control	**	NS	0	0	No[c]	0.57	0.40	0.00		0.00	0.00	0.00	0.00	0.00
--treated						0.27	0.00	0.00		0.00	0.00	0.00	0.00	0.00
size class 3, control	**	*	**	**	No[c]	1.27	1.57	1.32		1.00	0.77	0.08	0.00	0.00
--treated						1.13	0.68	0.30		0.42	0.17	0.05	0.00	0.00
size class 4, control	*	*	**	**	No[c]	0.70	1.18	1.53		1.45	1.63	1.00	0.33	0.00
--treated						0.80	0.58	1.13		1.20	0.97	0.48	0.28	0.00
size class 5, control	*	NS	*	NS	No[c]	0.38	0.53	0.78		0.92	1.30	0.58	0.23	0.05
--treated						0.28	0.28	0.23		0.73	0.68	0.33	0.20	0.00
Trichoptera														
Lepidostoma, control	*	NS	NS	*	No[c]	0.59	0.46	0.48		0.18	0.75	0.42	0.23	0.50
--treated						0.41	0.06	0.12		0.80	0.20	0.28	0.39	0.10
Palaegabus, control	**	NS	**	*	No[c]	0.53	0.18	0.66		0.79	1.14	0.00	0.26	0.35
--treated						0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
Rhyacophilia, control	NS	NS	NS	*	No[d]	0.59	0.83	0.73		1.01	1.04	0.00	0.85	0.00
--treated						0.52	0.54	0.79		0.67	0.76	0.76	0.87	0.26
size class 1, control	*	NS	**	**	No[c]	0.10	0.10	0.00		0.20	0.13	0.00	0.00	0.00
--treated						0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
size class 2, control	NS	NS	**	**	No[b]	0.37	0.70	0.58		0.70	0.57	0.00	0.18	0.00
--treated						0.28	0.42	0.28		0.17	0.28	0.10	0.05	0.00
size class 3, control	NS	*	NS	NS	No	0.00	0.22	0.05		0.35	0.68	0.23	0.38	0.20
--treated						0.07	0.00	0.12		0.05	0.35	0.27	0.40	0.23
size class 4, control	*	NS	*	NS	No[c]	0.00	0.00	0.00		0.35	0.10	0.05	0.10	0.05
--treated						0.12	0.07	0.07		0.05	0.13	0.00	0.00	0.00
size class 5, control	*	NS	*	NS	No	0.23	0.05	0.23		0.18	0.47	0.13	0.52	0.00
--treated						0.22	0.18	0.62		0.50	0.43	0.67	0.63	0.05
size class 6, control	NS	NS	*	NS	No[d]	0.18	0.23	0.22		0.00	0.08	0.00	0.05	0.00
--treated						0.12	0.12	0.07		0.13	0.10	0.15	0.10	0.00

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Table 3. Continued.

Diptera														
P. magnum, control	*	NS	NS	*	No	0.00	0.00	0.00		0.00	0.10	0.00	0.00	0.00
--treated						0.12	0.16	0.00		0.17	0.00	0.25	0.05	0.00
Prosimulium mixtum/fuscum, control	NS	NS	*	*	Yes	0.44	0.51	0.43		0.79	0.74	0.05	0.00	0.00
--treated						0.34	0.16	0.26		0.47	0.00	0.10	0.10	0.00
P. rhizophorum, control	NS	*	*	*	No[C]	0.01	0.38	0.29		0.52	0.48	0.05	0.00	0.00
--treated						0.26	0.06	0.26		0.29	0.00	0.10	0.10	0.00
S. vernum species group, control	NS	NS	**	NS	Yes	0.16	0.20	0.47		1.05	1.19	0.44	0.30	0.78
--treated						0.29	0.06	0.54		0.68	0.26	0.13	0.15	0.05
size class 1, control	NS	NS	NS	NS	No	0.10	0.00	0.00		0.00	0.38	0.13	0.00	0.00
--treated						0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00
size class 2, control	NS	NS	**	*	Yes	0.10	0.15	0.15		0.47	0.33	0.00	0.00	0.32
--treated						0.28	0.00	0.27		0.05	0.00	0.10	0.05	0.05
size class 3, control	NS	NS	**	NS	Yes	0.00	0.08	0.43		0.93	1.05	0.17	0.05	0.62
--treated						0.00	0.07	0.40		0.60	0.22	0.00	0.00	0.00
size class 4, control	NS	*	*	NS	No[C]	0.00	0.00	0.00		0.23	0.75	0.25	0.28	0.10
--treated						0.00	0.00	0.17		0.28	0.10	0.05	0.00	0.00
pupae, control	0	0	NS	*	No[D]	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.22
--treated						0.00	0.00	0.00		0.00	0.00	0.00	0.10	0.00
Similium vittatum, control	NS	NS	**	NS	Yes	0.00	0.00	0.00		0.15	0.15	0.05	0.00	0.10
--treated						0.00	0.00	0.06		0.00	0.00	0.00	0.00	0.00

Continued, next page

Table 3. Continued.

Stegopterna mutata, control	*	NS	**	**	No[c]	0.92	0.55	0.73		0.89	1.00	0.18	0.13	0.00
--treated						0.55	0.55	0.31		0.13	0.15	0.05	0.00	0.00
size class 2, control	NS	NS	NS	NS	No	0.00	0.05	0.05		0.10	0.00	0.00	0.00	0.00
--treated						0.00	0.07	0.00		0.00	0.00	0.00	0.00	0.00
size class 3, control	*	NS	**	**	No[c]	0.23	0.08	0.20		0.20	0.30	0.00	0.05	0.00
--treated						0.00	0.07	0.00		0.05	0.00	0.05	0.00	0.00
size class 4, control	*	NS	**	*	No[c]	0.82	0.45	0.40		0.45	0.48	0.18	0.08	0.00
--treated						0.37	0.38	0.07		0.00	0.10	0.00	0.00	0.00
pupae, control	NS	NS	**	**	No[b]	0.18	0.15	0.37		0.57	0.70	0.00	0.00	0.00
--treated						0.32	0.17	0.28		0.10	0.05	0.00	0.00	0.00
Ceratopogonidae, control	**	NS	**	NS	No	0.08	0.36	0.32		0.59	0.38	0.24	0.12	0.18
--treated						0.12	0.00	0.22		0.15	0.00	0.05	0.05	0.00
Chironomidae larvae, control	NS	NS	NS	*	No[d]	1.63	1.82	1.88		1.98	2.04	1.62	1.28	1.58
--treated						1.84	1.72	2.32		1.91	1.89	1.99	1.71	1.00
Chironomidae pupae, control	NS	**	NS	**	No[c]	0.37	0.55	0.21		0.67	0.77	0.53	0.38	0.28
--treated						0.12	0.22	0.84		0.52	0.17	1.01	0.75	0.13
Non-Insect Macroinvertebrates														
Annelidae, control	*	**	*	**	No[c]	0.36	0.42	0.18		0.72	0.20	0.15	0.10	0.22
--treated						0.74	0.16	1.17		0.44	0.00	1.47	0.74	0.20
Acari, control	*	NS	*	NS	No[c]	0.00	0.13	0.34		0.13	0.23	0.05	0.08	0.48
--treated						0.38	0.22	0.33		0.05	0.10	0.13	0.00	0.10

* Significant, $P \leq 0.05$; ** Significant, $P \leq 0.001$; [a] treated site did not decrease with time as did control.; [b] confounding effect before treatment signficant difference in a smaller size class; [c] confounding before treatment interaction/site differences; NS=not significantly different.

Table 4. Comparison of upstream control to downstream treated, drift samples.

Stream Control Drift vs Treated Drift	Before		After		Impact	Transformed Mean Abundance by Date					
						Before		After			
Taxa	Site	Site by Date Inter-action	Site	Site by Date Inter-action		5/5	5/13	5/21	5/23	5/28	6/2
Plecoptera											
Amphinemora, control	NS	*	NS	NS	No	0.09	0.14	0.14	0.29	0.10	0.27
--treated						0.35	0.11	0.33	0.20	0.49	0.43
size class 2, control	NS	NS	NS	NS	No	0.07	0.02	0.02	0.02	0.00	0.03
--treated						0.27	0.02	0.03	0.00	0.00	0.00
size class 3, control	NS	NS	NS	NS	No	0.03	0.13	0.12	0.25	0.02	0.08
--treated						0.15	0.10	0.28	0.10	0.17	0.13
size class 4, control	0	0	*	NS	No[d]	0.00	0.00	0.02	0.12	0.07	0.13
--treated						0.00	0.00	0.13	0.12	0.37	0.28
size class 5, control	0	0	*	NS	No[d]	0.00	0.00	0.00	0.00	0.02	0.05
--treated						0.00	0.00	0.02	0.00	0.17	0.25
size class 6, control	0	0	NS	NS	No	0.00	0.00	0.00	0.00	0.00	0.05
--treated						0.00	0.00	0.00	0.00	0.00	0.03
Leuctra Type A, control	*	**	**	NS	No[d]	0.81	0.38	0.74	0.55	0.69	0.92
--treated						0.61	1.20	0.28	0.26	0.26	0.22
size class 1, control	NS	NS	NS	NS	No	0.10	0.00	0.00	0.01	0.00	0.00
--treated						0.05	0.07	0.00	0.00	0.00	0.00
size class 2, control	NS	*	**	NS	No[c]	0.47	0.23	0.52	0.29	0.33	0.50
--treated						0.27	0.77	0.07	0.08	0.03	0.02
size class 3, control	*	**	**	NS	No[c]	0.62	0.25	0.48	0.37	0.57	0.80
--treated						0.47	0.95	0.23	0.22	0.22	0.20
size class 4, control	*	*	NS	NS	No[c]	0.03	0.03	0.02	0.06	0.05	0.07
--treated						0.05	0.33	0.03	0.02	0.02	0.02
size class 5, control	NS	NS	0	0	No	0.00	0.02	0.00	0.00	0.00	0.00
--treated						0.00	0.16	0.00	0.00	0.00	0.00

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Table 4. Continued.

Leuctra Type B, control	*	*	**	*	No[c]	0.06	0.22		0.18	0.11	0.25	0.43
--treated						0.05	0.55		0.09	0.02	0.07	0.05
size class 2, control	NS	NS	*	NS	No[a]	0.00	0.02		0.12	0.02	0.05	0.03
--treated						0.00	0.00		0.00	0.00	0.00	0.00
size class 3, control	*	*	**	*	No[c]	0.05	0.07		0.17	0.07	0.20	0.37
--treated						0.05	0.33		0.10	0.02	0.07	0.03
size class 4, control	NS	NS	NS	NS	No	0.02	0.15		0.02	0.03	0.02	0.02
--treated						0.00	0.38		0.00	0.00	0.02	0.02
Ostrocerca, control	NS	NS	NS	NS	No	1.13	1.04		1.01	0.93	0.36	0.36
--treated						1.18	1.41		0.74	0.66	0.50	0.39
size class 2, control	NS	NS	0	0	No	0.13	0.02		0.00	0.00	0.00	0.00
--treated						0.08	0.05		0.00	0.00	0.00	0.00
size class 3, control	*	*	**	NS	No[c]	0.97	0.38		0.30	0.18	0.02	0.00
--treated						0.93	0.90		0.12	0.07	0.00	0.00
size class 4, control	NS	NS	NS	NS	No	0.58	0.82		0.82	0.75	0.28	0.25
--treated						0.72	1.12		0.53	0.42	0.33	0.28
size class 5, control	*	NS	NS	NS	No	0.37	0.60		0.58	0.65	0.12	0.18
--treated						0.57	0.85		0.50	0.42	0.30	0.20
size class 6, control	0	0	NS	NS	No	0.00	0.00		0.00	0.00	0.00	0.00
--treated						0.00	0.00		0.00	0.03	0.00	0.02
Trichoptera												
Lepidostoma, control	NS	NS	NS	NS	No	0.25	0.19		0.13	0.15	0.16	0.38
--treated						0.14	0.33		0.23	0.04	0.24	0.38

Continued, next page

Table 4. Continued.

Rhyacophilia, control	NS	*	NS	NS	No	0.31	0.20		0.20	0.27	0.32	0.21
--treated						0.12	0.46		0.24	0.19	0.20	0.28
size class 2, control	NS	*	NS	NS	No	0.08	0.00		0.02	0.02	0.02	0.03
--treated						0.00	0.15		0.00	0.00	0.00	0.00
size class 3, control	NS	NS	NS	*	No[D]	0.10	0.03		0.00	0.03	0.07	0.00
--treated						0.02	0.00		0.02	0.00	0.00	0.00
size class 4, control	NS	NS	NS	NS	No	0.02	0.02		0.03	0.12	0.03	0.12
--treated						0.03	0.10		0.02	0.00	0.05	0.07
size class 5, control	NS	NS	NS	NS	No	0.12	0.17		0.15	0.13	0.23	0.12
--treated						0.10	0.32		0.22	0.17	0.17	0.20
size class 6, control	NS	NS	NS	NS	No	0.03	0.03		0.03	0.02	0.03	0.00
--treated						0.00	0.02		0.02	0.02	0.03	0.08
Diptera												
P. magnum, control	**	NS	*	NS	No	0.01	0.03		0.05	0.02	0.02	0.01
--treated						0.37	0.45		0.41	0.27	0.08	0.20
size class 3, control	*	NS	NS	NS	No	0.00	0.00		0.00	0.00	0.02	0.00
--treated						0.13	0.02		0.02	0.00	0.00	0.00
size class 4, control	**	NS	NS	NS	No	0.02	0.07		0.00	0.02	0.00	0.00
--treated						0.28	0.20		0.02	0.02	0.00	0.00
size class 5, control	**	*	**	NS	No	0.00	0.00		0.05	0.00	0.00	0.02
--treated						0.03	0.22		0.40	0.28	0.08	0.03
pupae, control	NS	NS	NS	NS	No	0.00	0.00		0.00	0.00	0.00	0.00
--treated						0.00	0.00		0.00	0.00	0.00	0.17

Continued, next page

Table 4. Continued.

Prosimulium mixtum/fuscum, control	NS	NS	**	NS	Yes	0.37	0.26		0.14	0.16	0.11	0.15
--treated						0.32	0.14		0.09	0.00	0.02	0.00
size class 1, control	NS	NS	0	0	No	0.02	0.02		0.00	0.00	0.00	0.00
--treated						0.00	0.00		0.00	0.00	0.00	0.00
size class 2, control	NS	NS	NS	NS	No	0.08	0.02		0.00	0.00	0.00	0.02
--treated						0.02	0.02		0.00	0.00	0.00	0.00
size class 3, control	NS	NS	NS	NS	No	0.13	0.05		0.10	0.10	0.00	0.00
--treated						0.12	0.02		0.03	0.00	0.00	0.00
size class 4, control	NS	NS	**	NS	Yes	0.20	0.15		0.07	0.10	0.12	0.15
--treated						0.25	0.03		0.02	0.00	0.00	0.00
pupae, control	NS	NS	NS	NS	No	0.00	0.15		0.00	0.00	0.02	0.00
--treated						0.05	0.03		0.02	0.00	0.02	0.00
P. rhizophorum, control	NS	NS	NS	*	No[d]	0.10	0.08		0.12	0.00	0.00	0.03
--treated						0.11	0.17		0.02	0.04	0.02	0.00
S. vernum species group--control	NS	NS	**	NS	Yes	0.29	0.58		0.53	0.70	0.52	0.68
--treated						0.25	0.31		0.32	0.06	0.13	0.09
size class 1, control	NS	NS	*	NS	Yes	0.23	0.12		0.03	0.25	0.05	0.07
--treated						0.07	0.00		0.00	0.00	0.00	0.00
size class 2, control	NS	*	*	NS	No[c]	0.13	0.50		0.07	0.15	0.10	0.37
--treated						0.20	0.08		0.00	0.00	0.08	0.05
size class 3, control	NS	*	**	NS	No[c]	0.00	0.43		0.47	0.52	0.12	0.40
--treated						0.02	0.20		0.22	0.00	0.07	0.05
size class 4, control	NS	NS	**	NS	No[b]	0.00	0.00		0.18	0.37	0.42	0.37
--treated						0.00	0.02		0.17	0.07	0.08	0.00
pupae, control	0	0	NS	*	No[b]	0.00	0.00		0.00	0.00	0.02	0.27
--treated						0.00	0.00		0.00	0.00	0.00	0.00
Similium vittatum, control	0	0	*	*	Yes	0.00	0.00		0.02	0.00	0.02	0.17
--treated						0.00	0.00		0.02	0.00	0.00	0.00

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Table 4. Continued.

Stegopterna mutata, control	*	NS	**	NS	No[c]	0.74	1.01		0.21	0.29	0.06	0.18
--treated						0.44	0.34		0.06	0.05	0.00	0.00
size class 1, control	NS	NS	0	0	No	0.00	0.03		0.00	0.00	0.00	0.00
--treated						0.00	0.00		0.00	0.00	0.00	0.00
size class 2, control	**	**	NS	NS	No	0.00	0.27		0.00	0.02	0.00	0.00
--treated						0.00	0.00		0.00	0.00	0.00	0.00
size class 3, control	*	NS	*	NS	No[c]	0.18	0.25		0.05	0.07	0.02	0.02
--treated						0.07	0.03		0.02	0.00	0.00	0.00
size class 4, control	**	NS	**	NS	No[c]	0.63	0.80		0.07	0.18	0.02	0.15
--treated						0.37	0.20		0.02	0.00	0.00	0.00
size class 5, control	NS	NS	0	0	No	0.02	0.00		0.00	0.00	0.00	0.00
--treated						0.00	0.00		0.00	0.00	0.00	0.00
pupae, control	*	*	*	NS	No[c]	0.17	0.82		0.13	0.10	0.03	0.03
--treated						0.10	0.15		0.02	0.07	0.00	0.00
Ceratopogonidae, control	NS	NS	NS	NS	No	0.10	0.00		0.00	0.00	0.02	0.00
--treated						0.00	0.00		0.00	115	0.00	0.00
Chironomidae larvae, control	NS	NS	NS	NS	No	0.60	0.64		0.62	0.58	0.20	0.38
--treated						0.65	0.91		1.99	0.65	0.35	0.40
Chironomidae pupae, control	NS	NS	*	*	No[d]	0.57	0.05		0.29	0.15	0.63	0.96
--treated						0.65	0.82		0.18	0.26	1.40	0.93

* Significant, $P \leq 0.05$; ** Significant, $P \leq 0.001$; [a] treated site did not decrease with time as did control; [b] confounding effect before treatment signficant difference in a smaller size class; [c] confounding before treatment interaction/site differences; [d] not present in treatment site before spray; NS=not significantly different.

Fig. 1. Site of diflubenzuron treatment in Warwick, Massachusetts.

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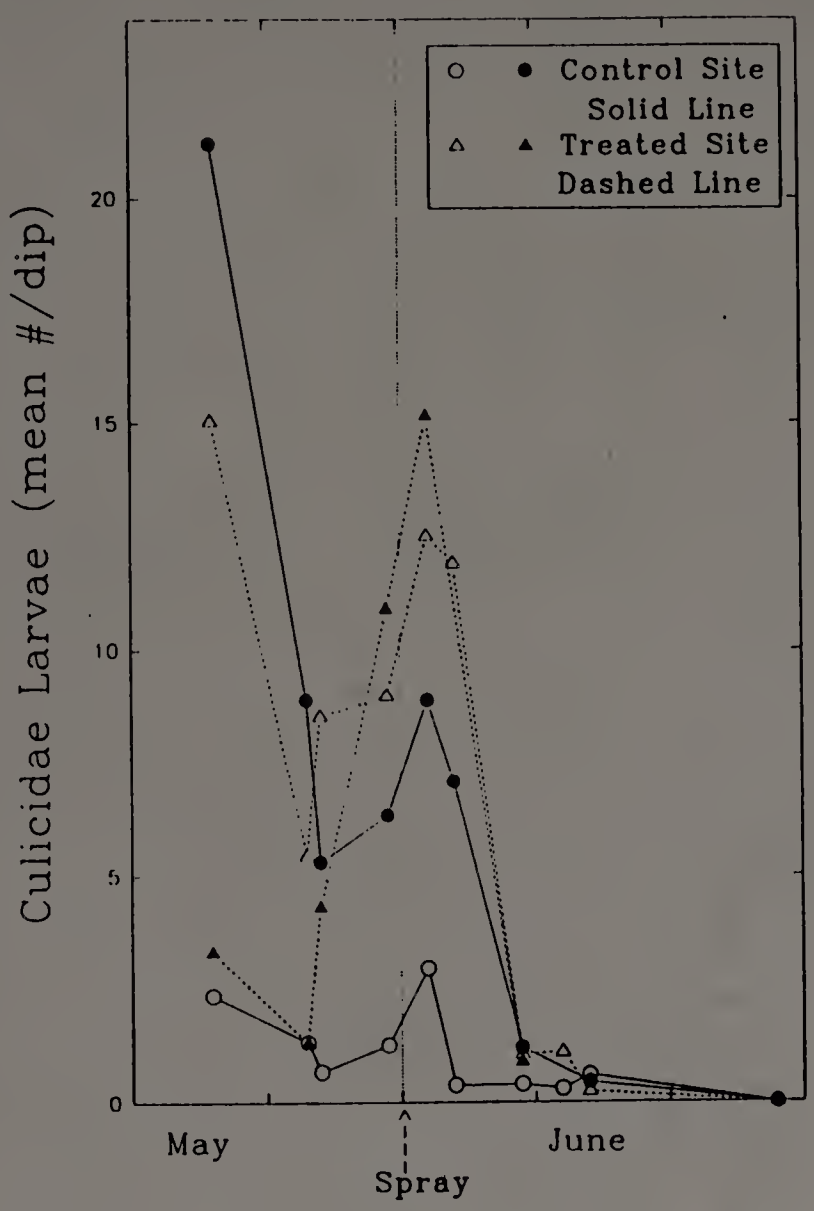
Fig. 2. Culicidae mean abundance by vernal pool with time, control site (circles), treated site (triangles), VP1 site (open symbols), VP2 site (closed symbols), larvae (A), pupae (B).


A
Culicidae Larvae (mean #/dip)
Control Site
Solid Line
Treated Site
Dashed Line
20
15
10
5
0
May
Spray
June

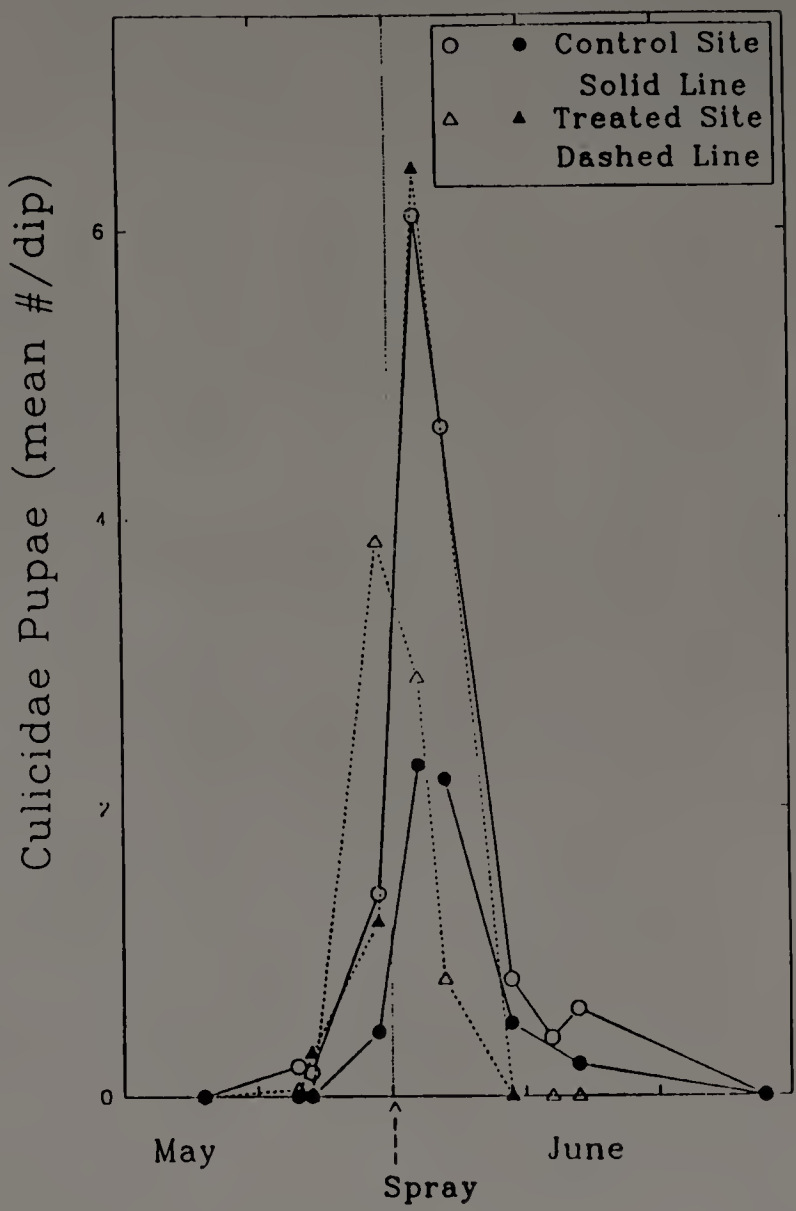
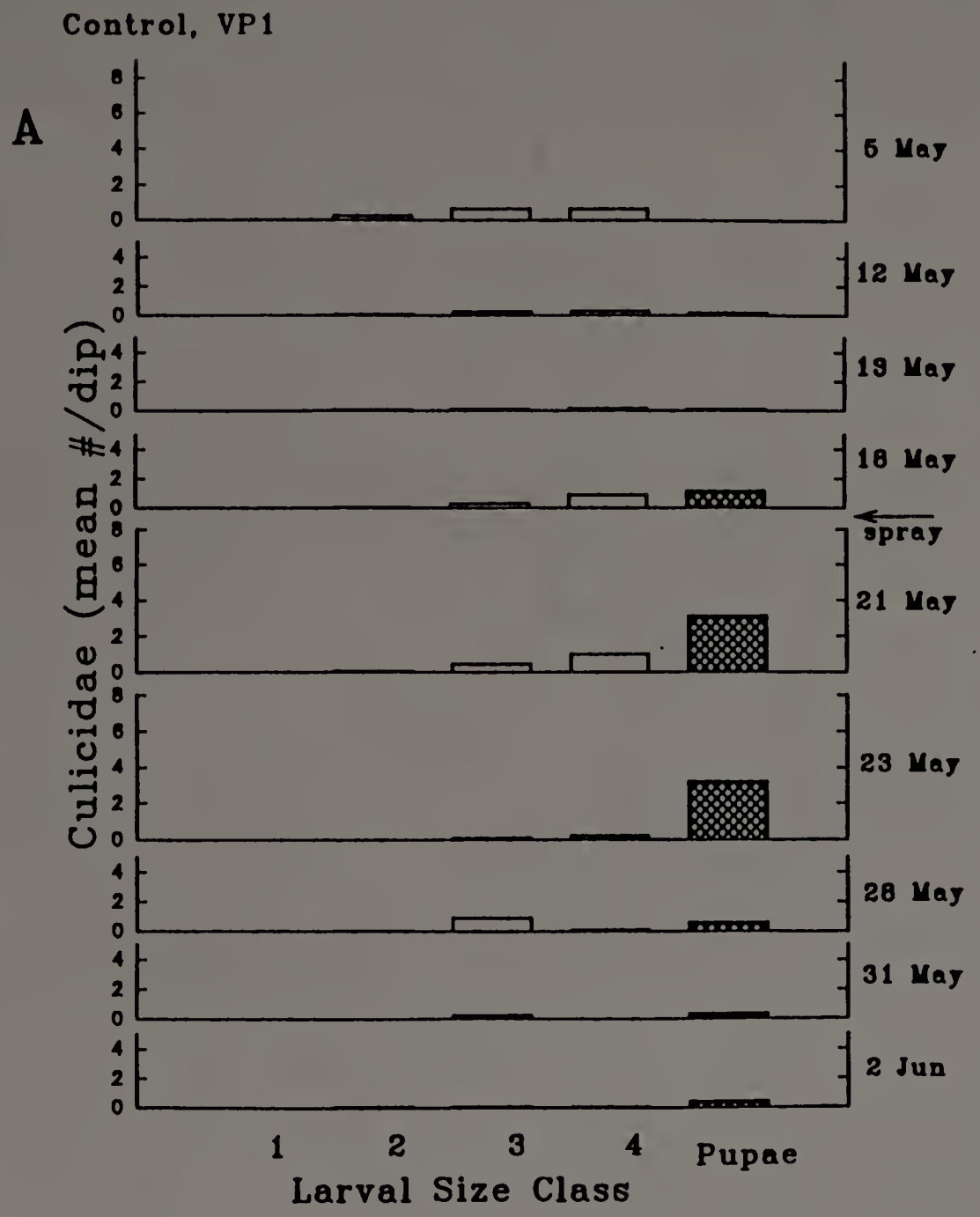

B
Culicidae Pupae (mean #/dip)
Control Site
Solid Line
Treated Site
Dashed Line
6
4
2
0
May
Spray
June

Fig. 3. Culicidae mean abundance by size class with time, larvae (open bars), pupae (hatched bars), control VP1 site (A), control VP2 site (B).


Control, VP1
A
5 May
12 May
13 May
18 May
spray
21 May
23 May
28 May
31 May
2 Jun
Culicidae (mean #/dip)
1
2
3
4
Pupae
Larval Size Class

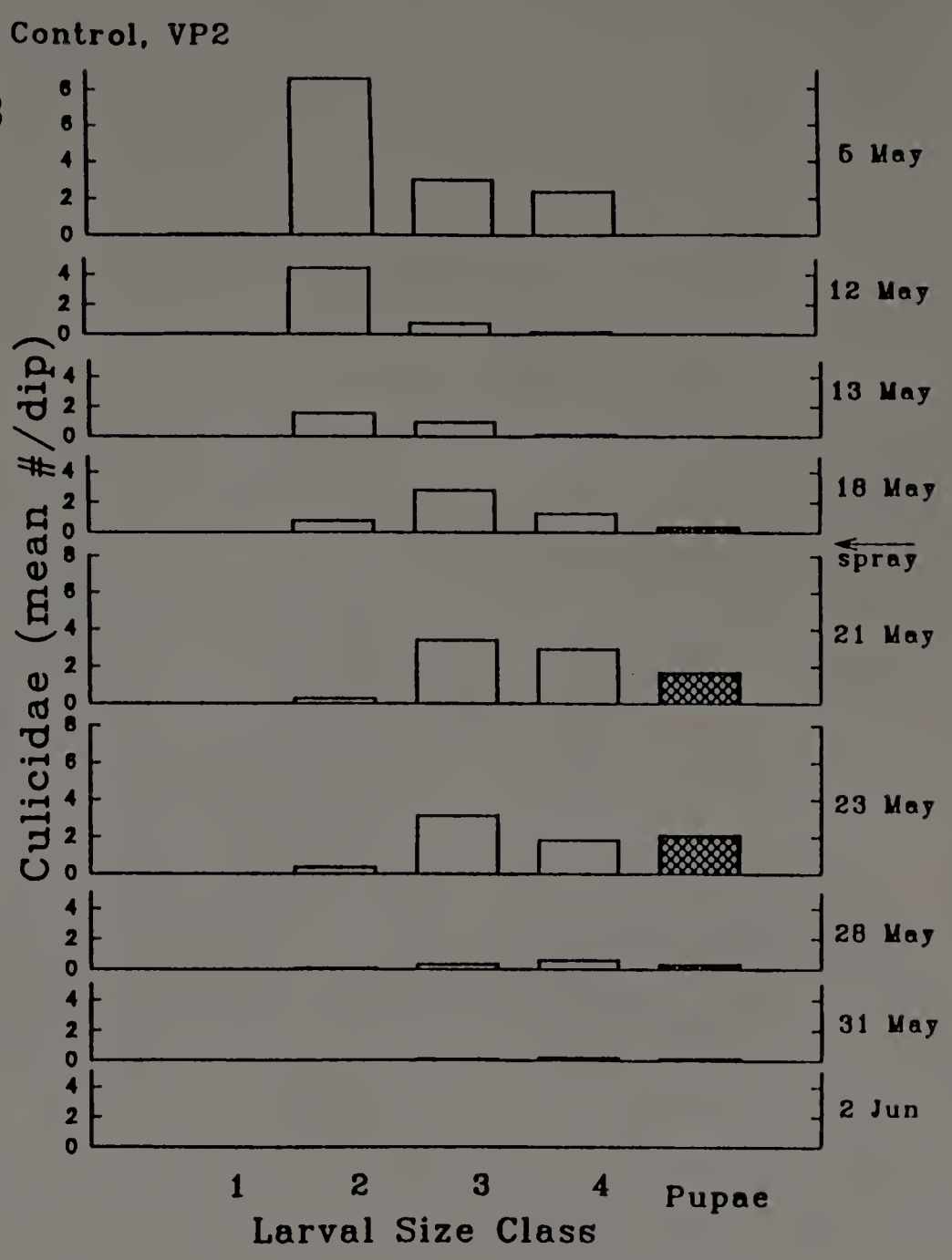
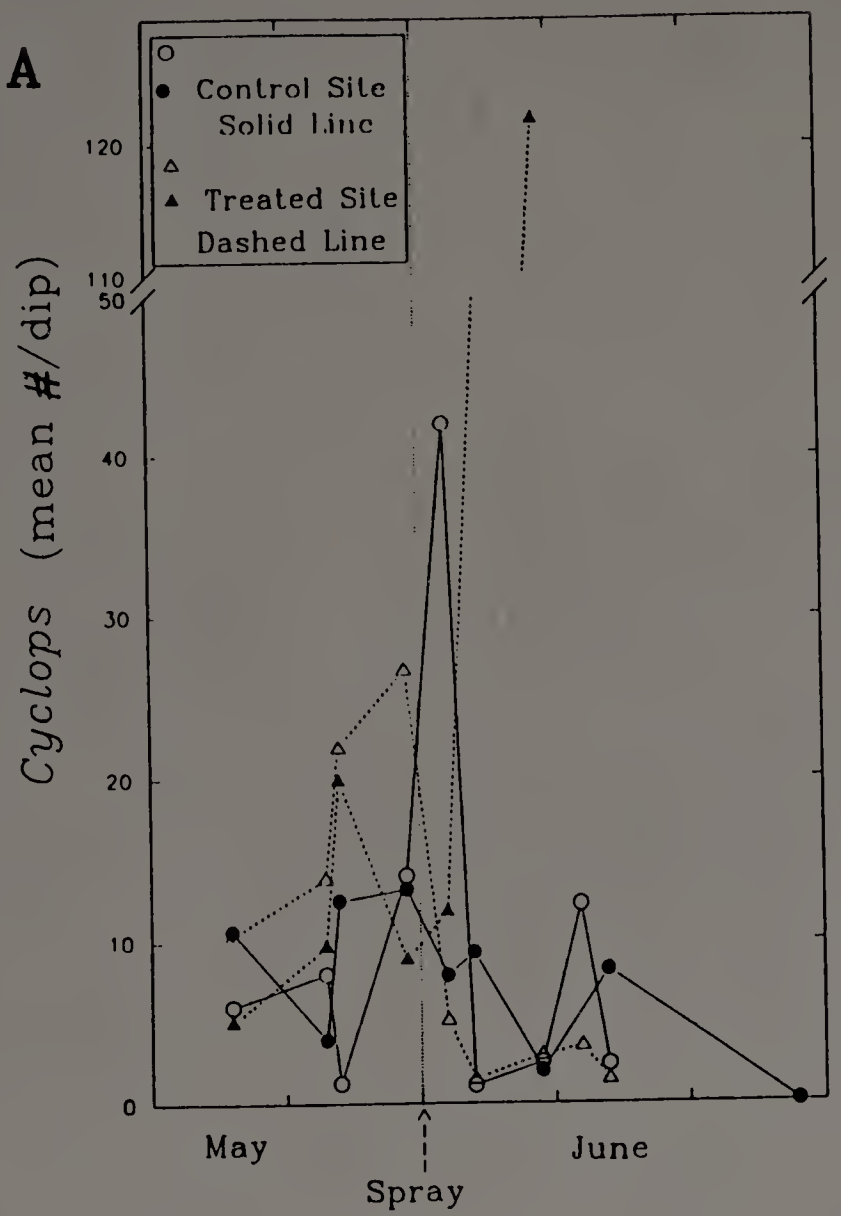

Control, VP2
B
5 May
12 May
13 May
18 May
spray
21 May
23 May
28 May
31 May
2 Jun
Culicidae (mean #/dip)
1
2
3
4
Pupae
Larval Size Class

Fig. 4. Culicidae mean abundance by size class with time, larvae (open bars), pupae (hatched bars), treated VP1 site.


Treated Site
Culicidae (mean #/dip)
0
2
4
6
8
5 May
12 May
13 May
18 May
spray
21 May
23 May
28 May
31 May
2 Jun
1
2
3
4
Pupae
Larval Size Class

Fig. 5. *Cyclops* mean abundance by vernal pool with time (A), and Acari mean abundance by vernal pool with time (B); control site (circles), treated site (triangles), VP1 site (open symbols), VP2 site (closed symbols).


A
Control Site
Solid Line
Treated Site
Dashed Line
Cyclops (mean #/dip)
120
110
50
40
30
20
10
0
May
Spray
June

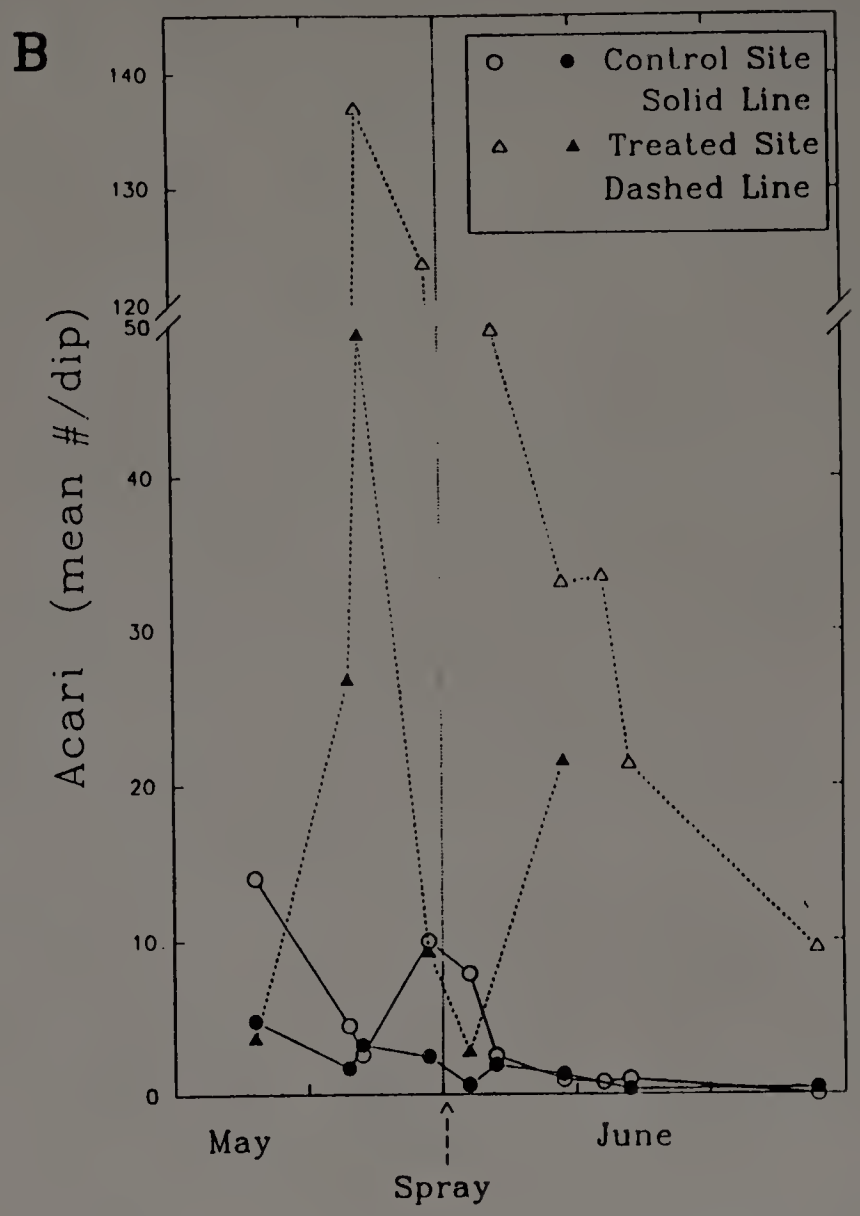
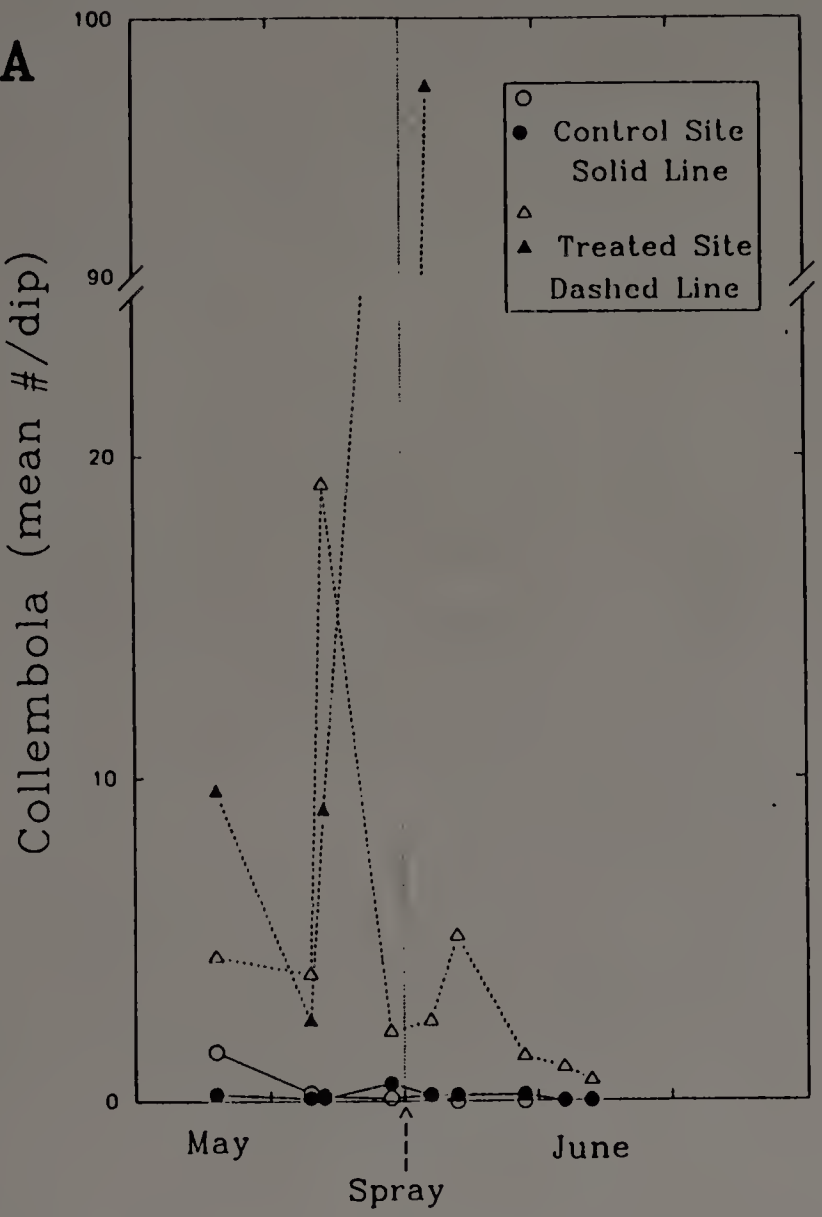

B
Control Site
Solid Line
Treated Site
Dashed Line
Acari (mean #/dip)
140
130
120
50
40
30
20
10
0
May
Spray
June

Fig. 6. Collembola mean abundance by vernal pool with time (A), and Tardigrada mean abundance by vernal pool with time (B); control site (circles), treated site (triangles), VP1 site (open symbols), VP2 site (closed symbols).


A
Collembola (mean #/dip)
100
90
20
10
0
Control Site
Solid Line
Treated Site
Dashed Line
May
June
Spray

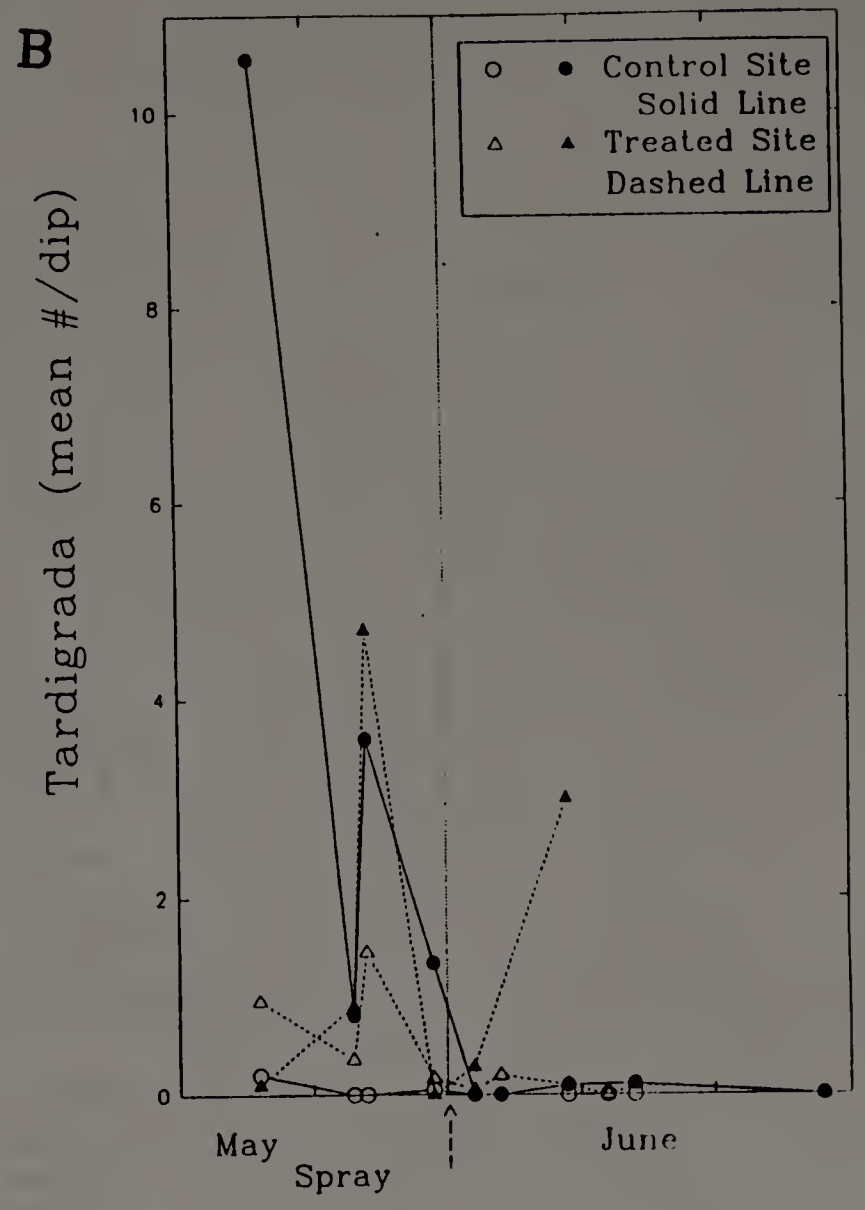
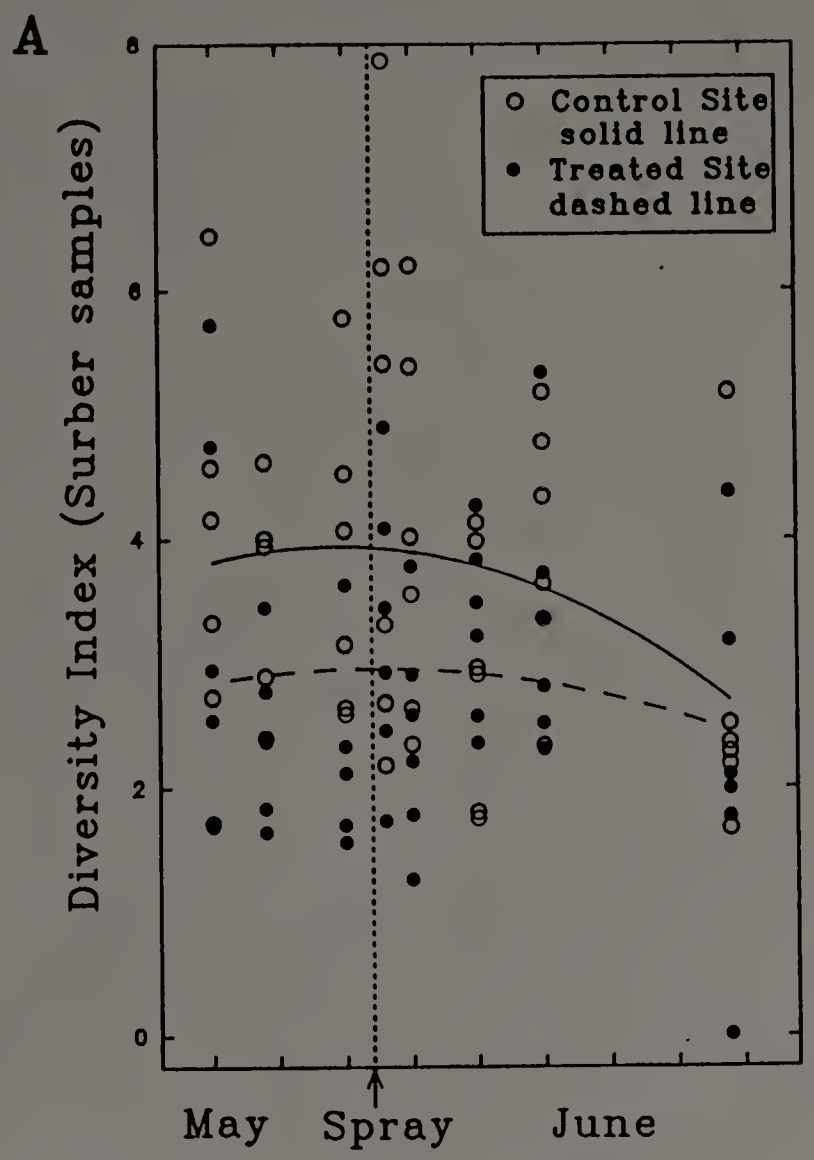

B
Tardigrada (mean #/dip)
10
8
6
4
2
0
Control Site
Solid Line
Treated Site
Dashed Line
May
Spray
June

Fig. 7. Stream diversity index by site with time, Surber samples (A), drift samples (B).


A
Diversity Index (Surber samples)
Control Site
solid line
Treated Site
dashed line
0
2
4
6
8
May
Spray
June

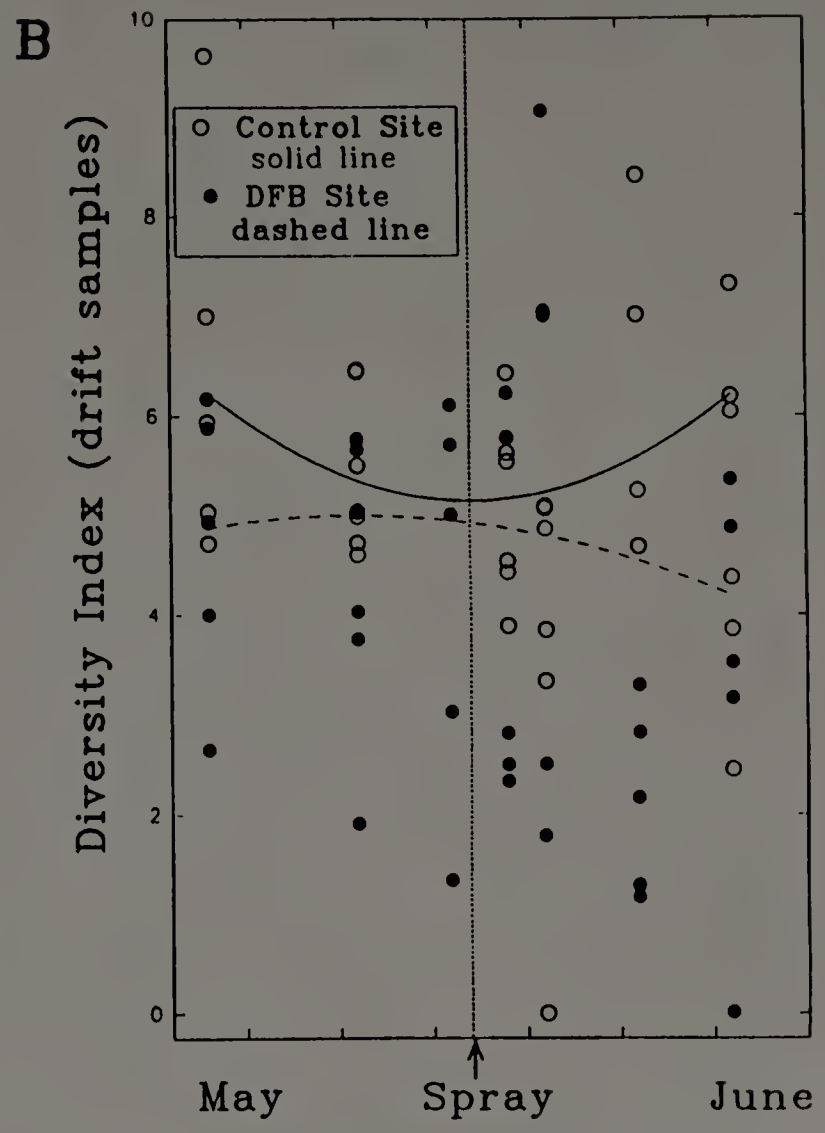
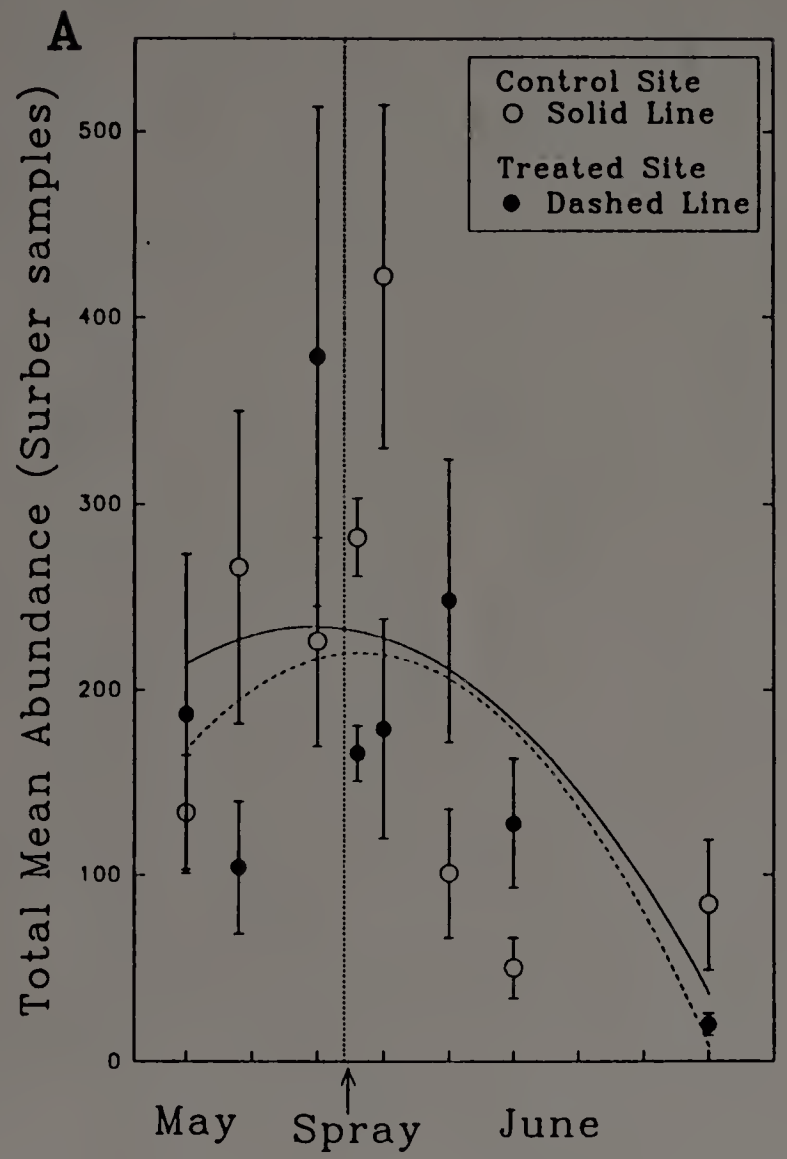

B
Diversity Index (drift samples)
Control Site
solid line
DFB Site
dashed line
0
2
4
6
8
10
May
Spray
June

Fig. 8. Stream total abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Control Site
O Solid Line
Treated Site
● Dashed Line
Total Mean Abundance (Surber samples)
500
400
300
200
100
0
May
Spray
June

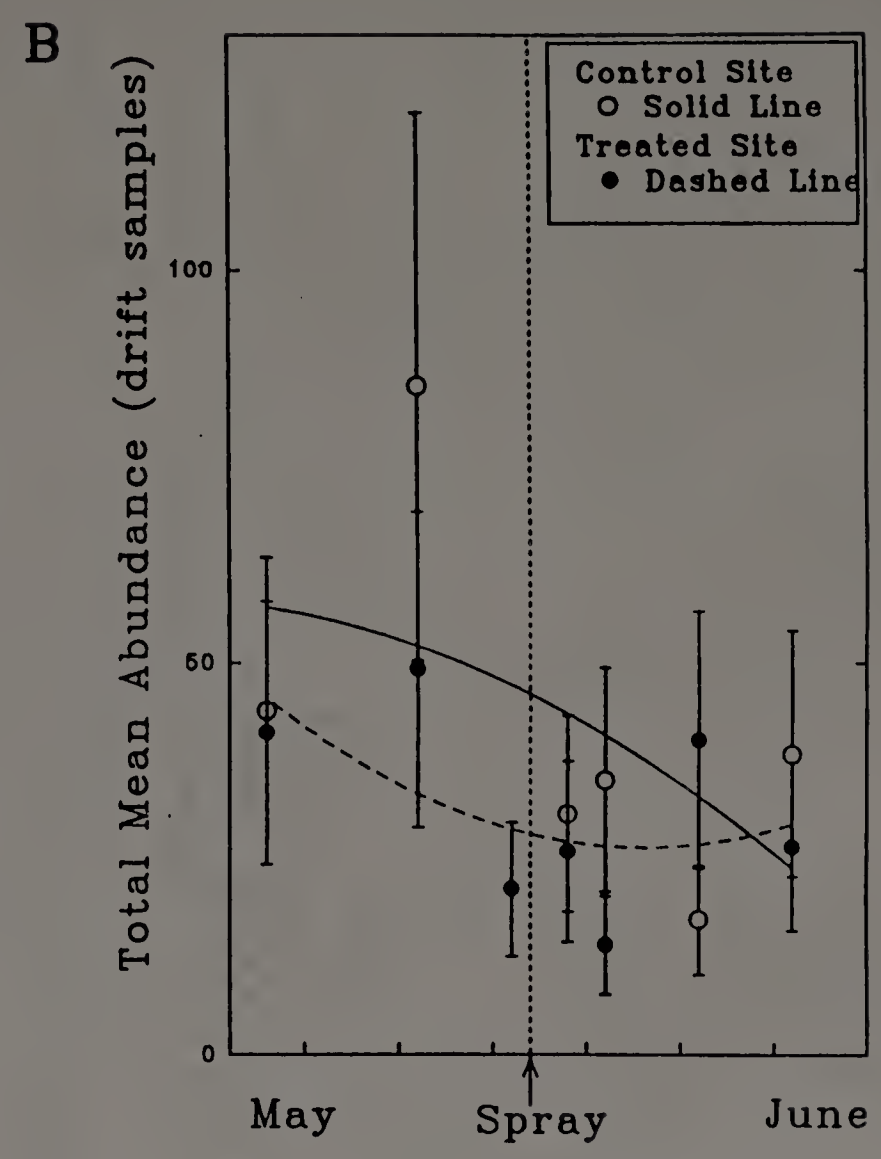
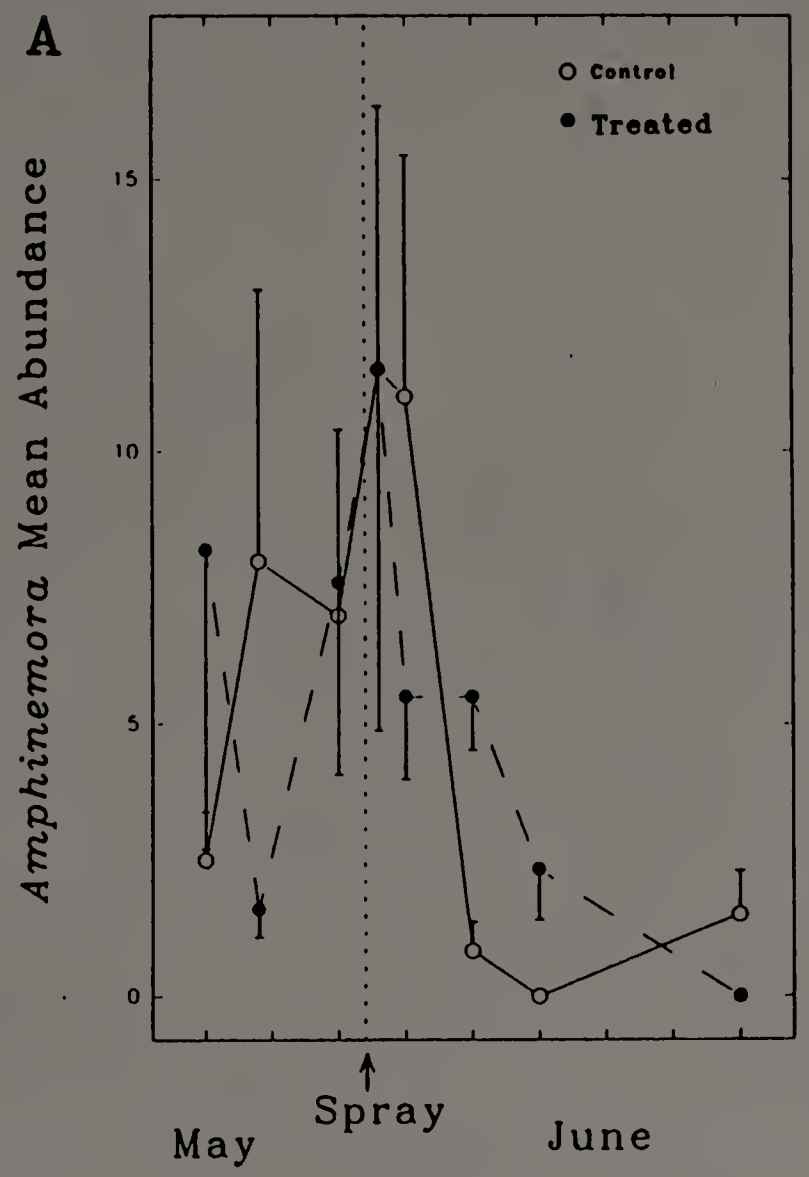

B
Control Site
O Solid Line
Treated Site
● Dashed Line
Total Mean Abundance (drift samples)
100
50
0
May
Spray
June

Fig. 9. *Amphinemora* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Amphinemoura Mean Abundance
15
10
5
0
Control
Treated
May
Spray
June

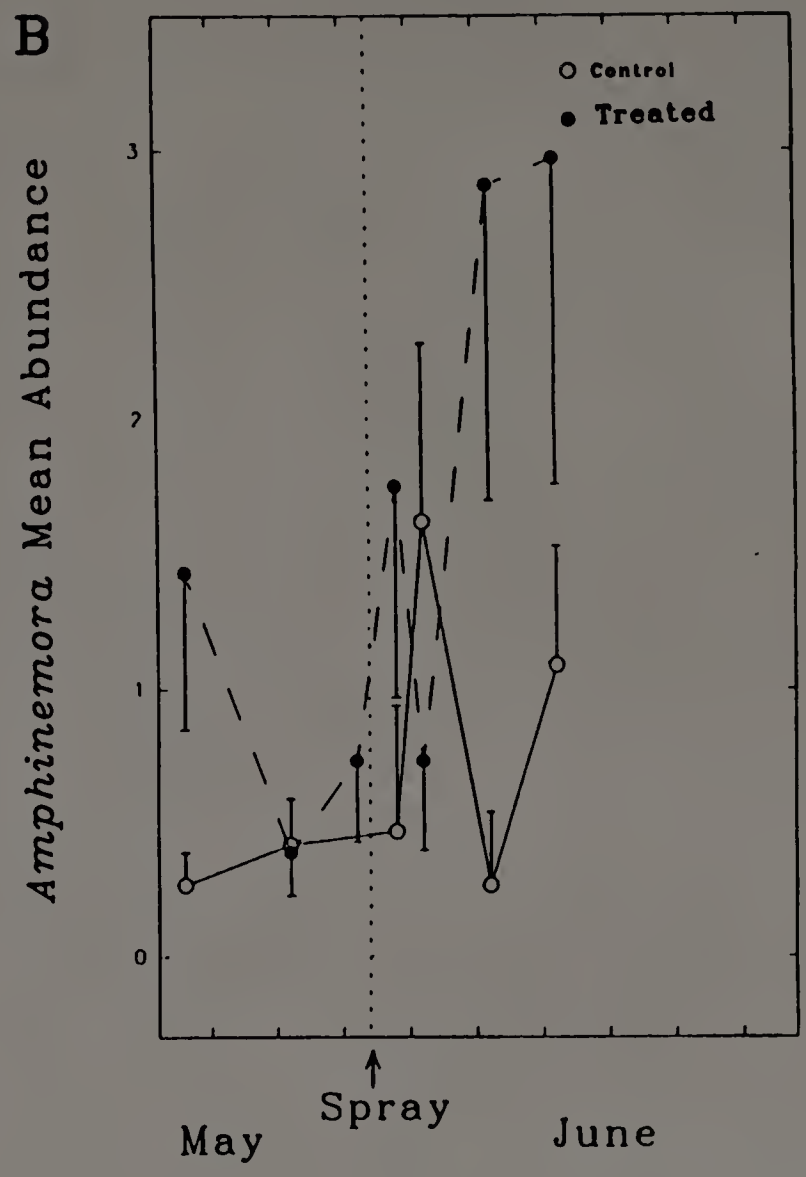
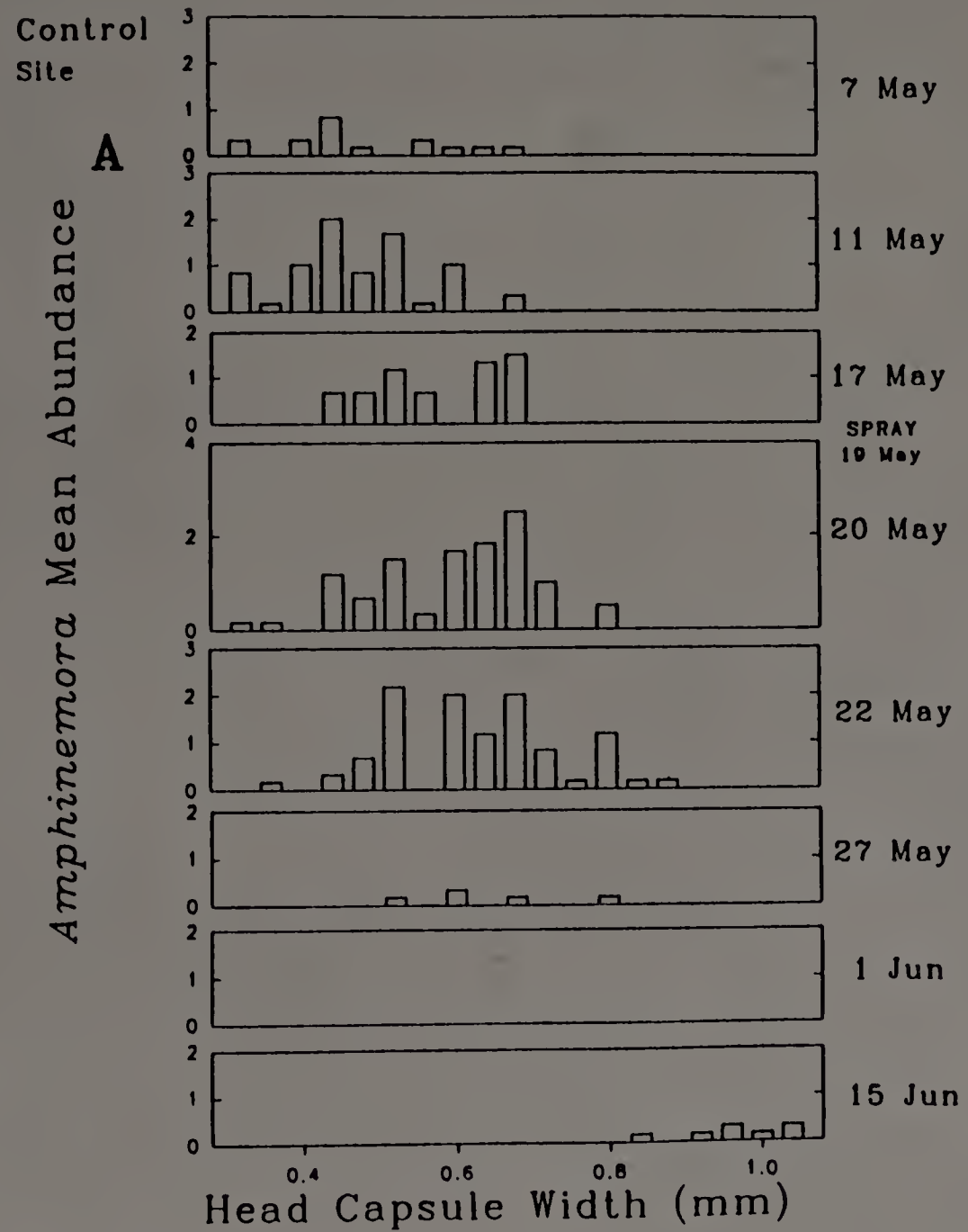

B
Amphinemoura Mean Abundance
3
2
1
0
Control
Treated
May
Spray
June

Fig. 10. *Amphinemora* mean abundance in Surber samples by size class with time, control (A), treated site (B).


Control
Site
A
Amphinemoura Mean Abundance
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.4
0.6
0.8
1.0
Head Capsule Width (mm)

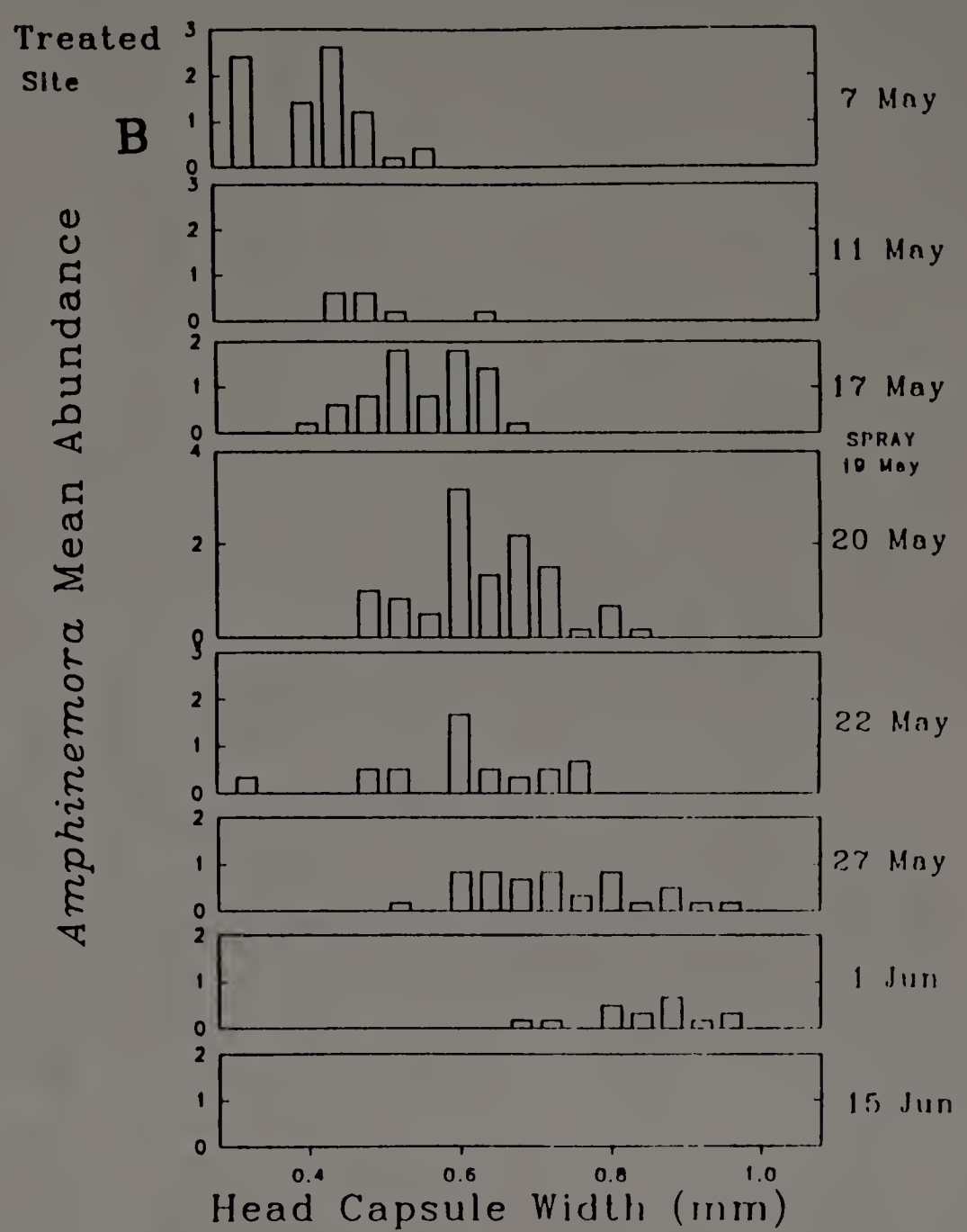

Treated
Site
B
Amphinemoura Mean Abundance
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.4
0.6
0.8
1.0
Head Capsule Width (mm)

Fig. 11. *Leuctra* Type A mean abundance by site with time, ± 1 S.E., drift samples.

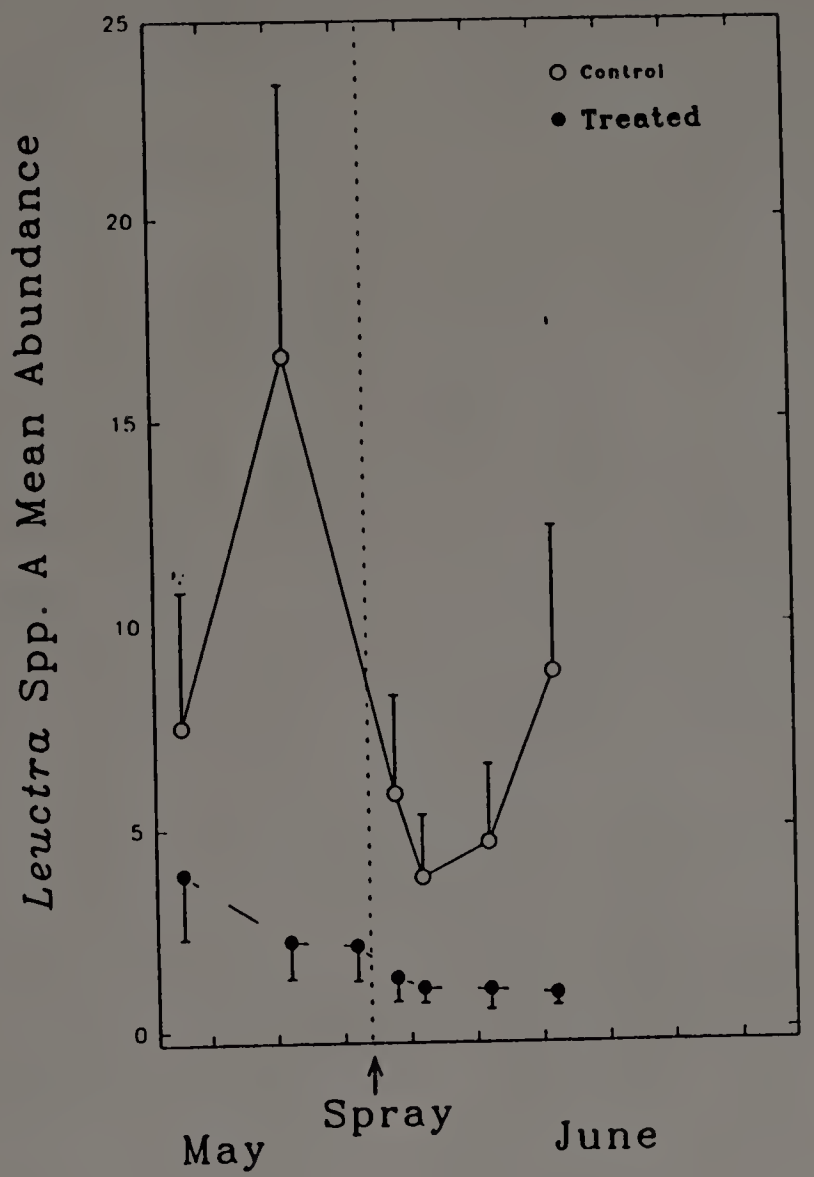
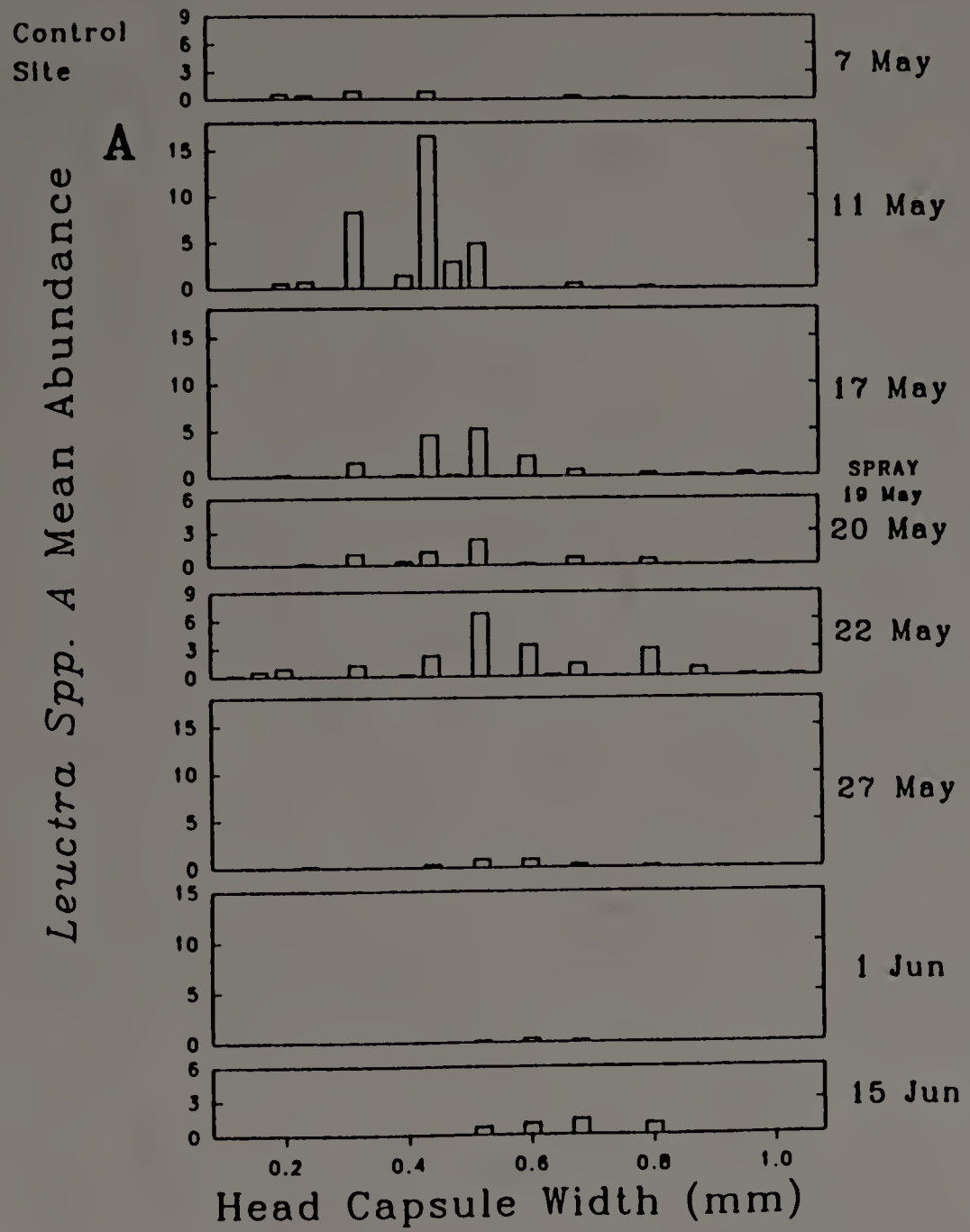

Leuctra Spp. A Mean Abundance
25
20
15
10
5
0
Control
Treated
Spray
May
June

Fig. 12. *Leuctra* type A mean abundance in Surber samples by size class with time, control (A), treated site (B).


Control Site
A
Leuctra Spp. A Mean Abundance
7 May
11 May
17 May
SPRAY 19 May
20 May
22 May
27 May
1 Jun
15 Jun
Head Capsule Width (mm)

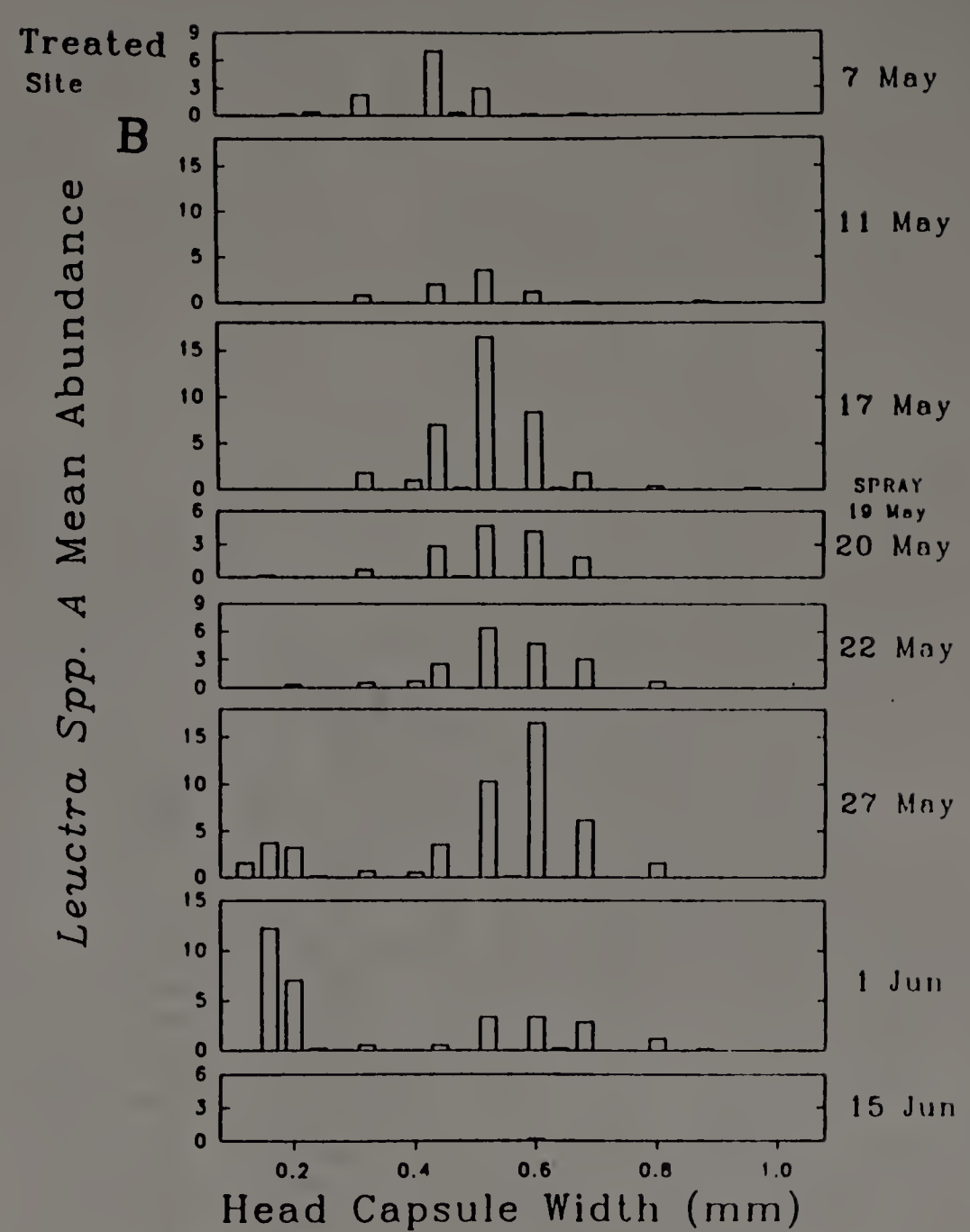
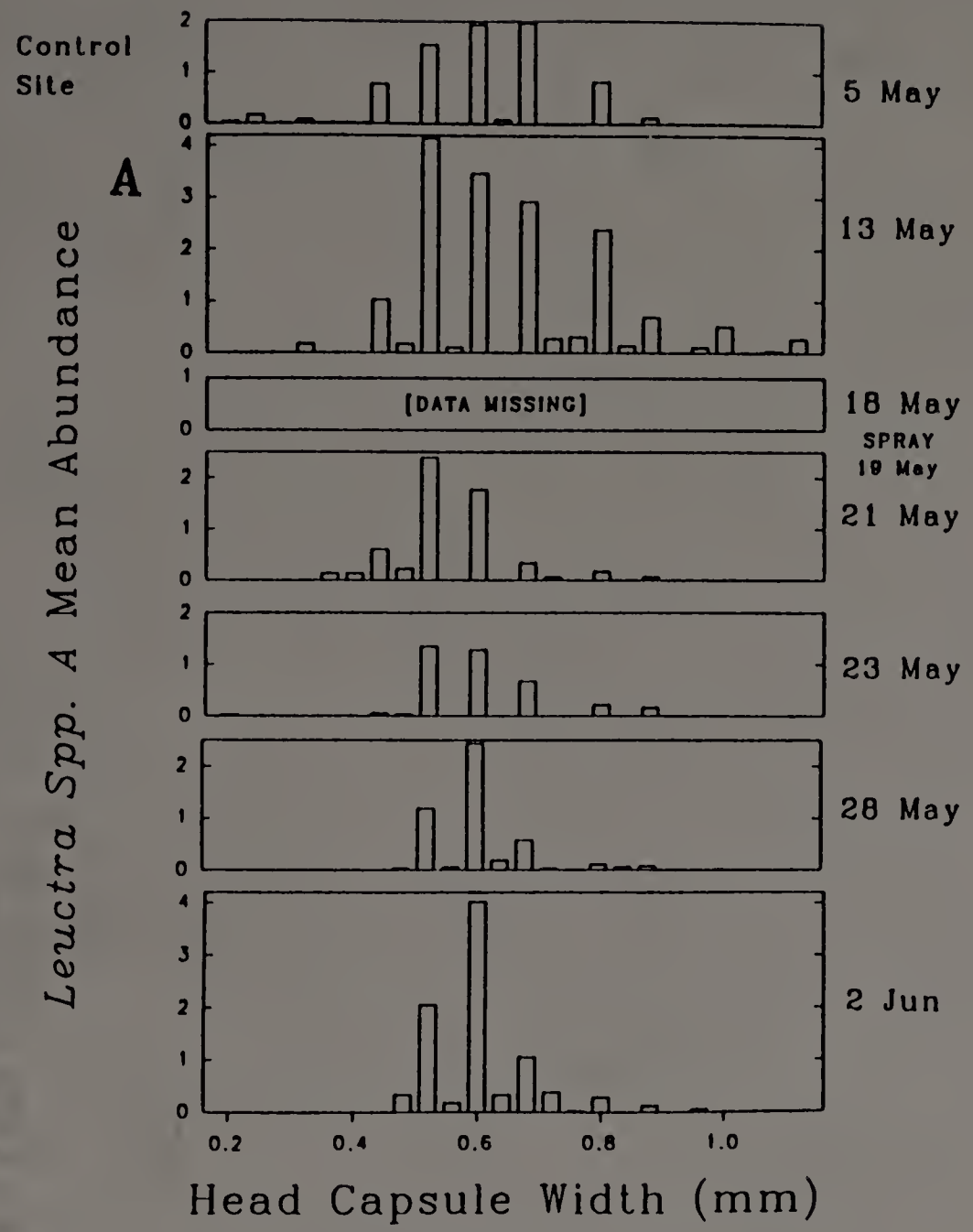

Treated Site
B
Leuctra Spp. A Mean Abundance
7 May
11 May
17 May
SPRAY 19 May
20 May
22 May
27 May
1 Jun
15 Jun
Head Capsule Width (mm)

Fig. 13. *Leuctra* type A mean abundance in drift samples by size class with time, control (A), treated site (B).


Control Site
A
Leuctra Spp. A Mean Abundance
5 May
13 May
[DATA MISSING]
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Head Capsule Width (mm)

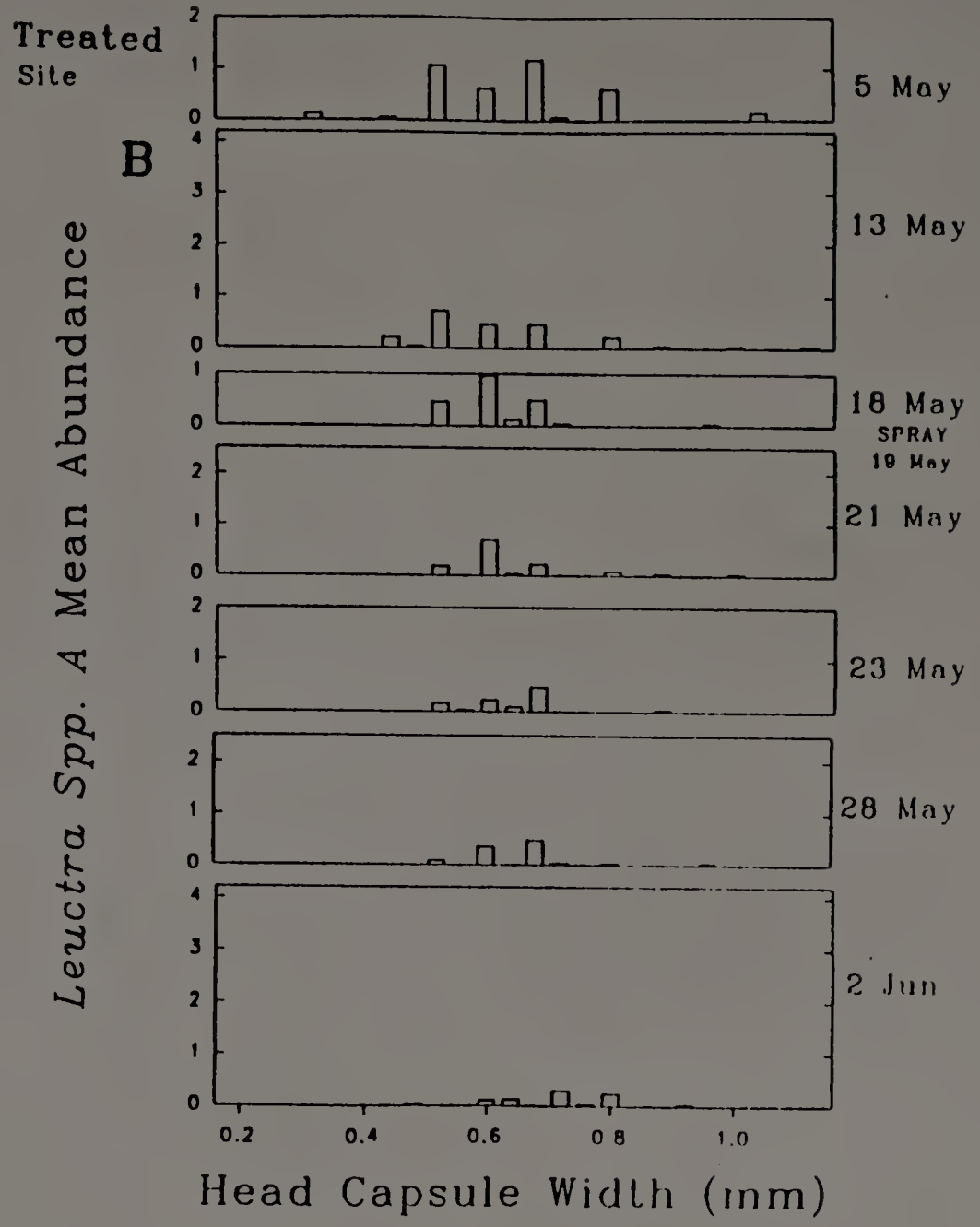
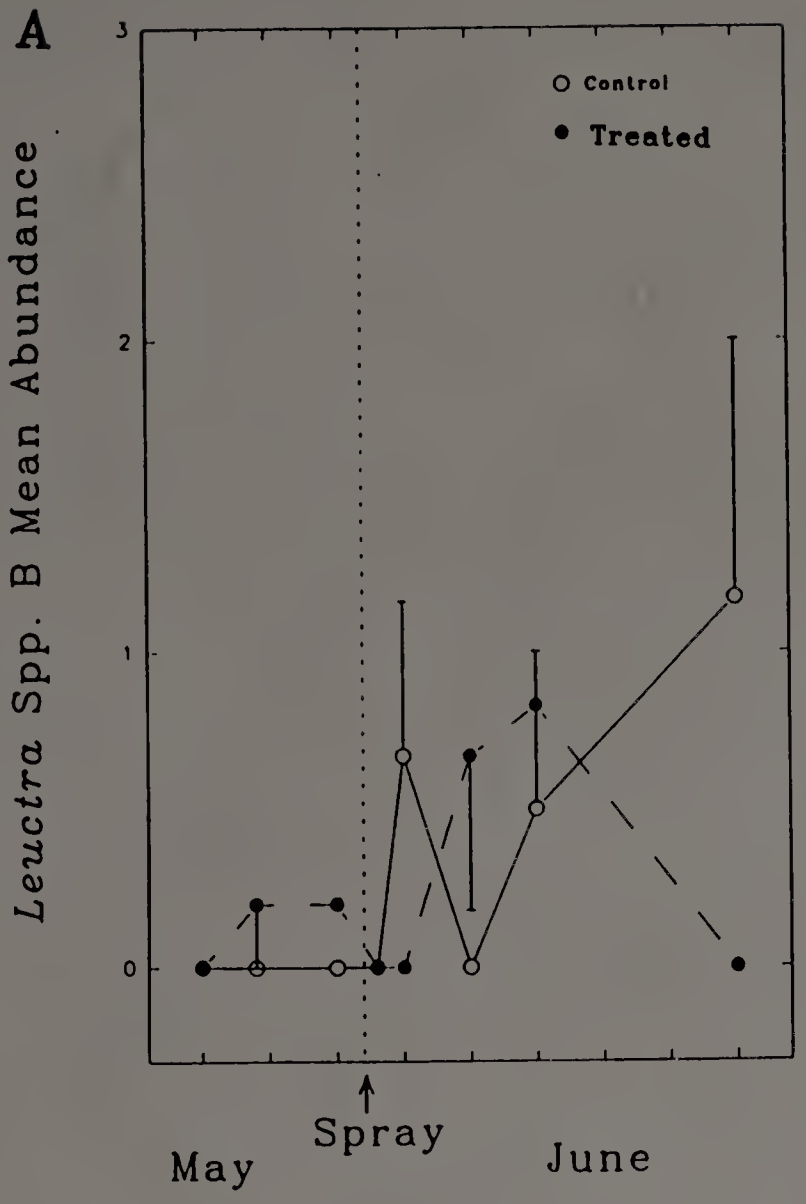

Treated Site
B
Leuctra Spp. A Mean Abundance
5 May
13 May
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Head Capsule Width (mm)

Fig. 14. *Leuctra* Type B mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Leuctra Spp. B Mean Abundance
3
2
1
0
Control
Treated
Spray
May
June

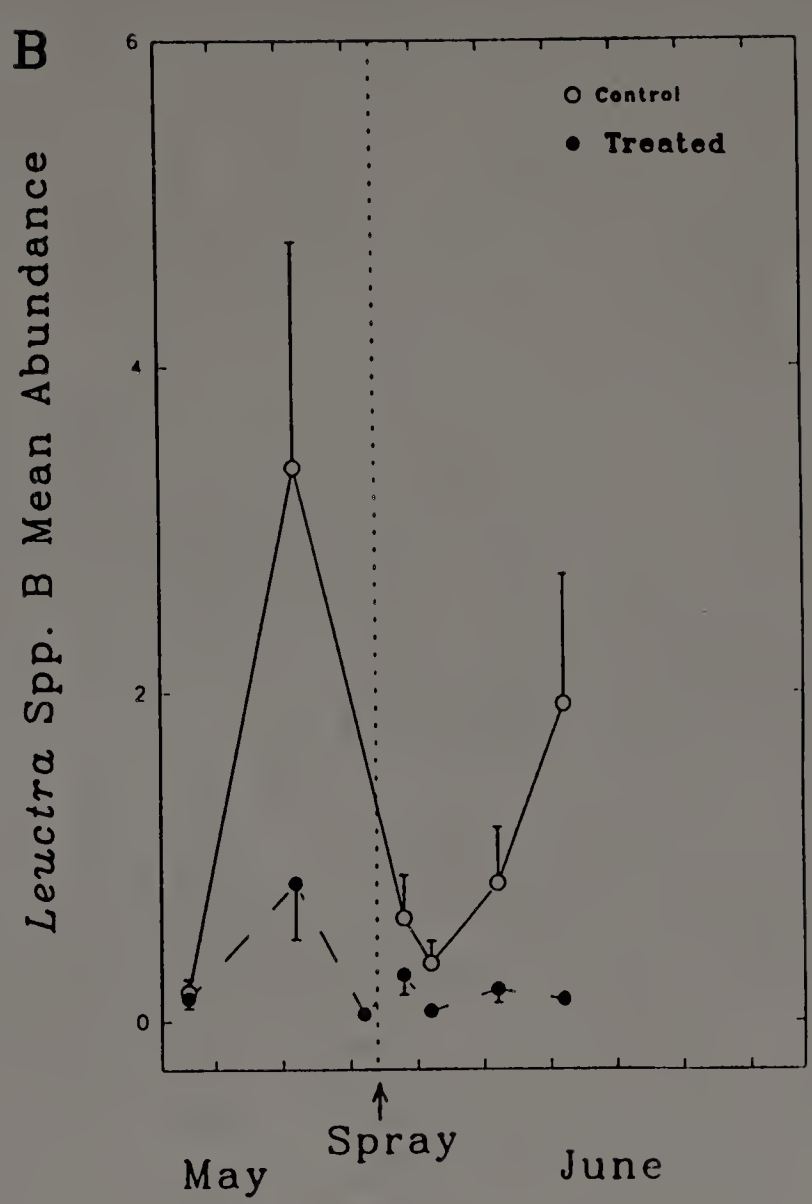
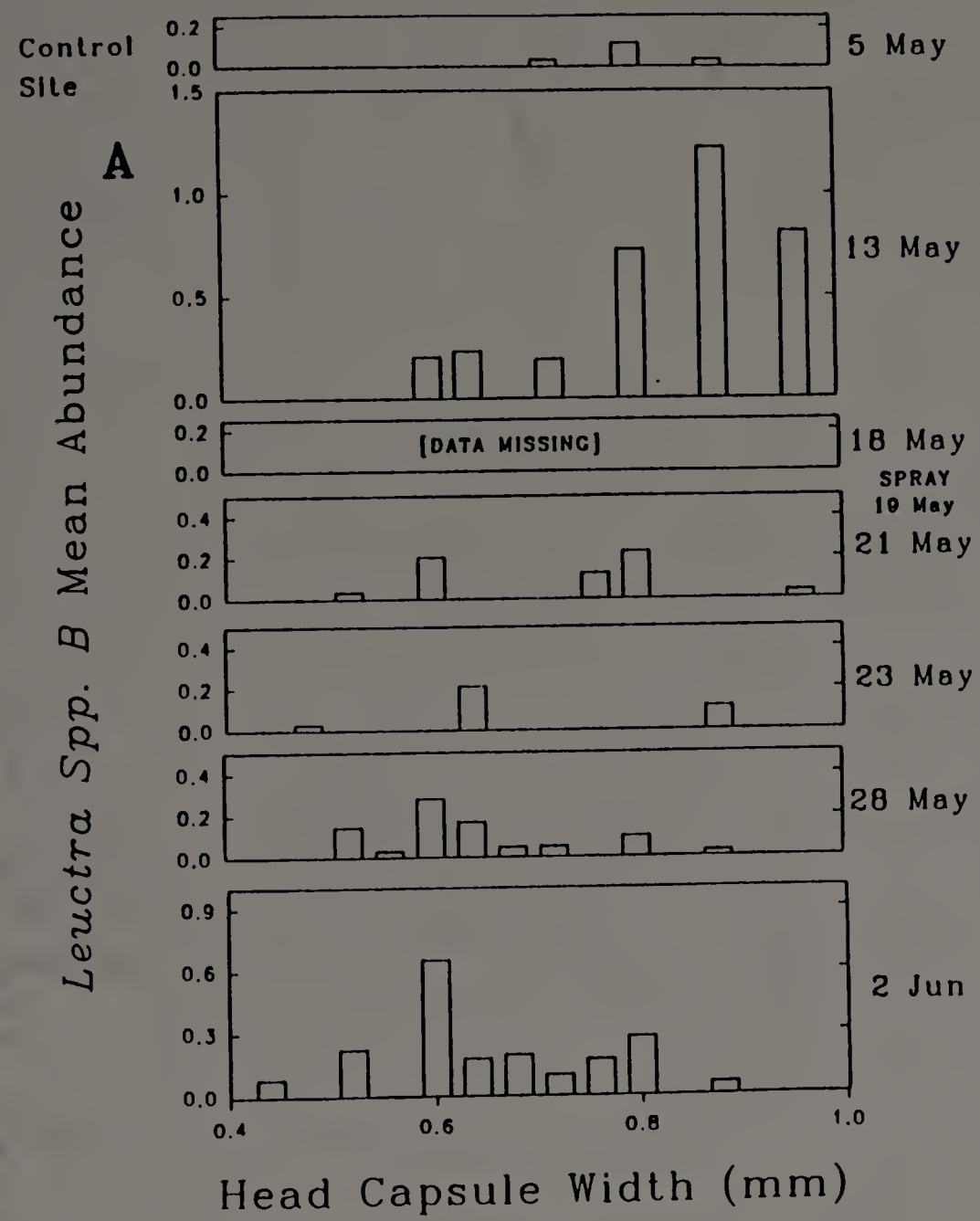

B
Leuctra Spp. B Mean Abundance
6
4
2
0
Control
Treated
Spray
May
June

Fig. 15. *Leuctra* type B mean abundance in drift samples by size class with time, control (A), treated site (B).


Control Site
A
Mean Abundance
Leuctra Spp. Bodies
5 May
13 May
[DATA MISSING]
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Head Capsule Width (mm)

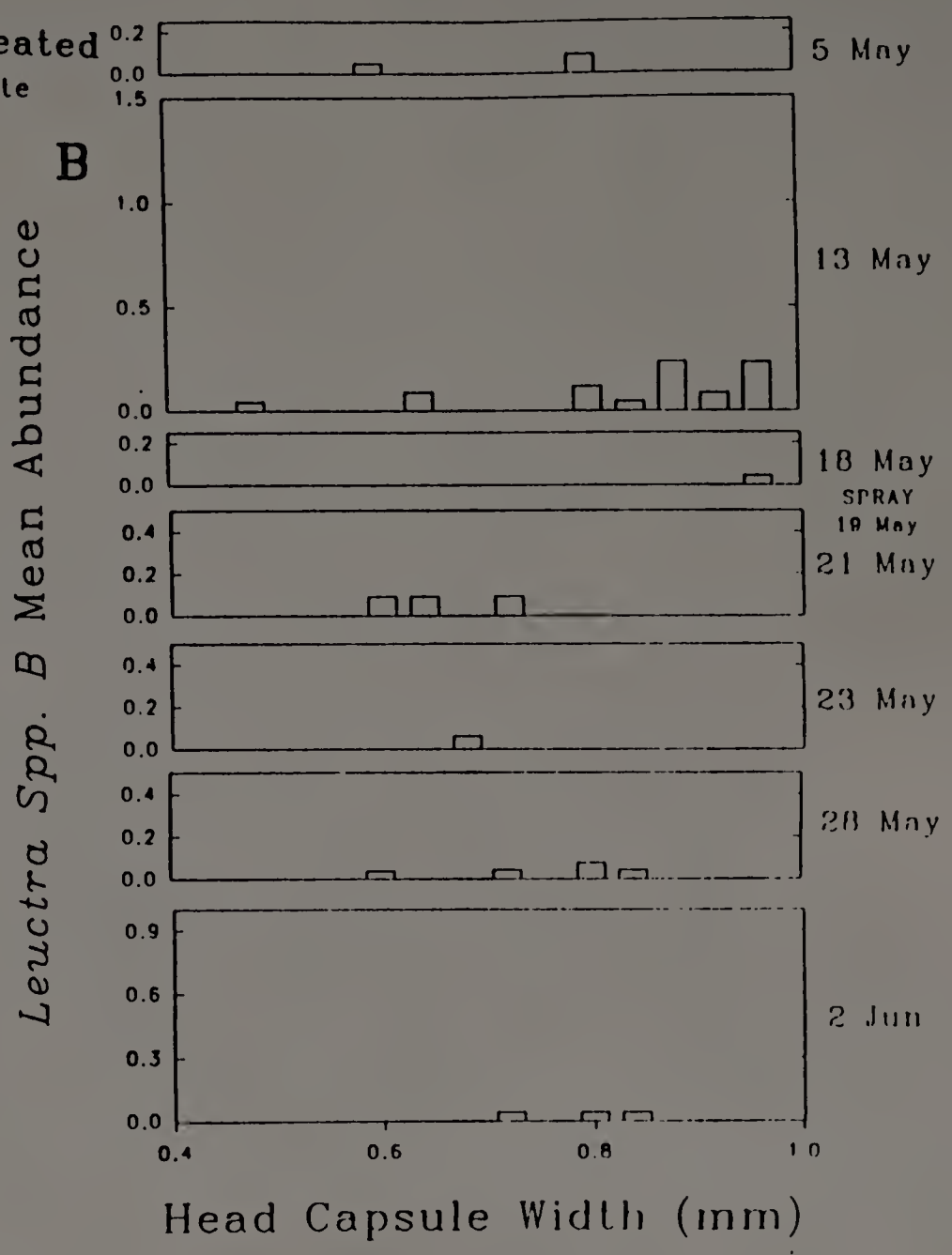
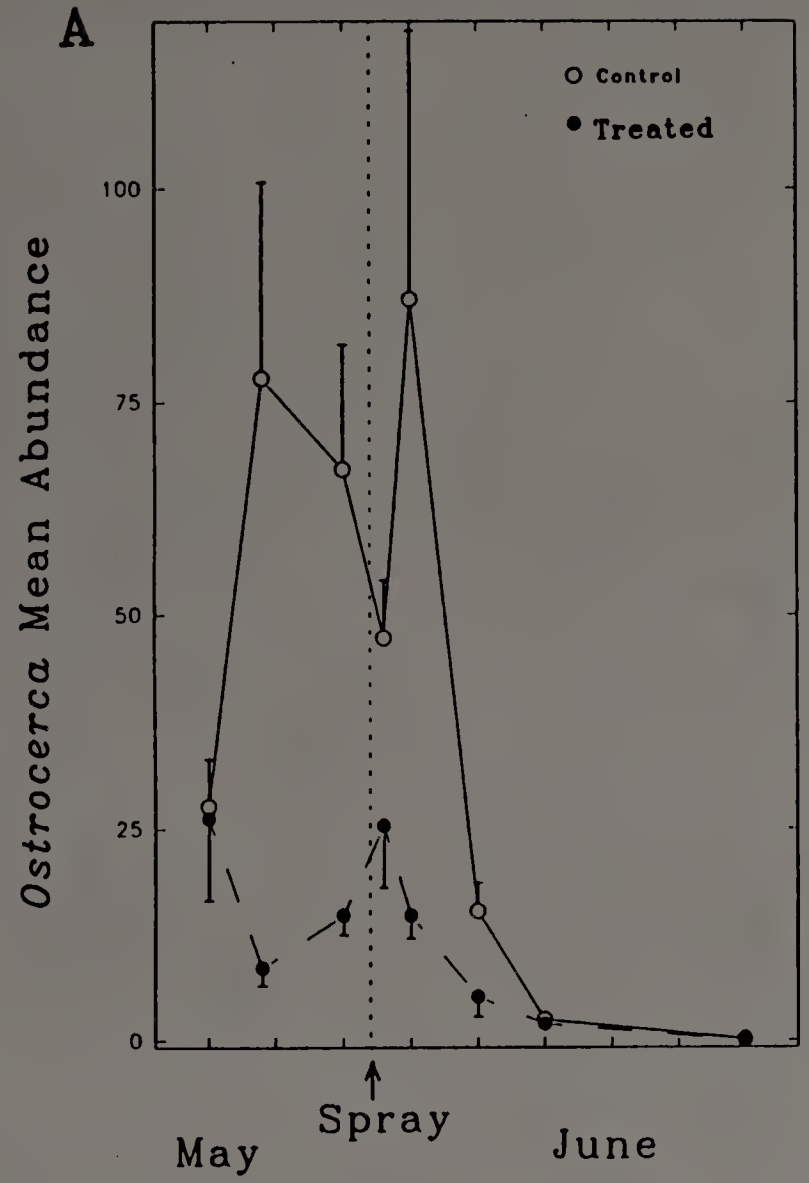

Treated Site
B
Mean Abundance
Leuctra Spp. Bodies
5 May
13 May
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Head Capsule Width (mm)

Fig. 16. *Ostrocerca* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Control
Treated
Ostrocerca Mean Abundance
100
75
50
25
0
Spray
May
June

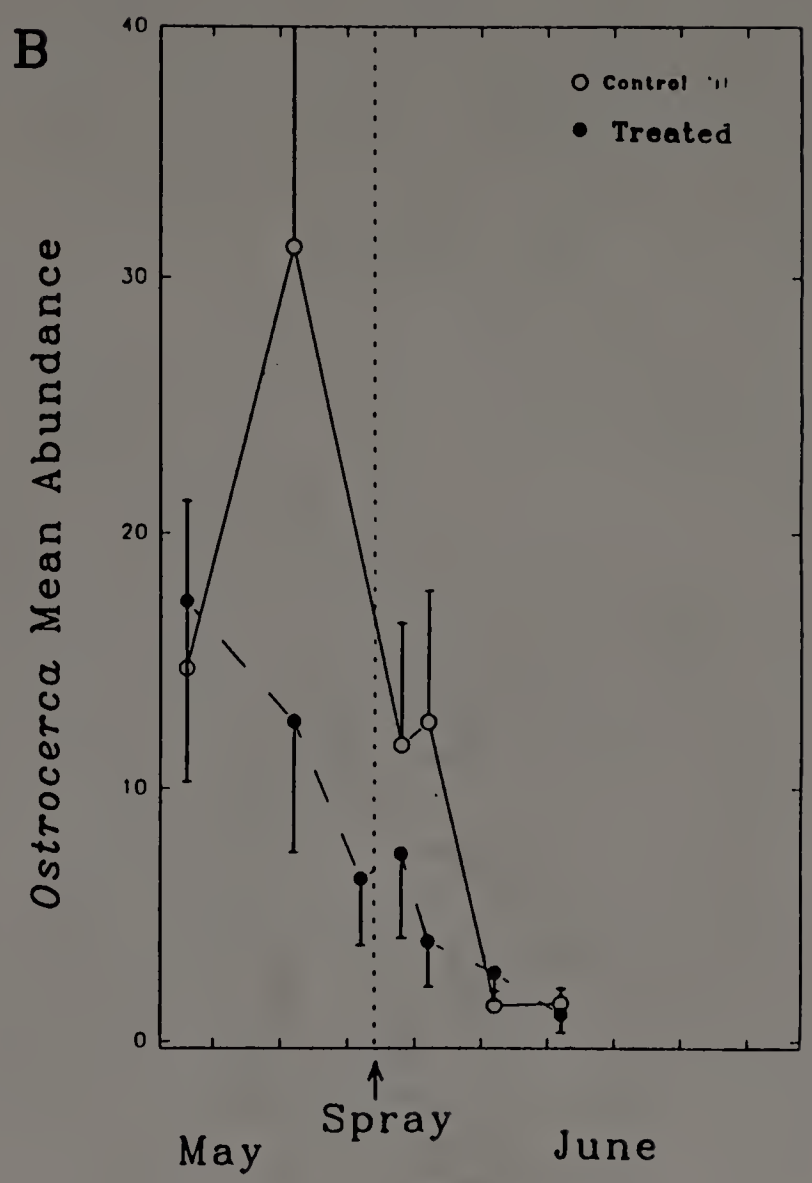
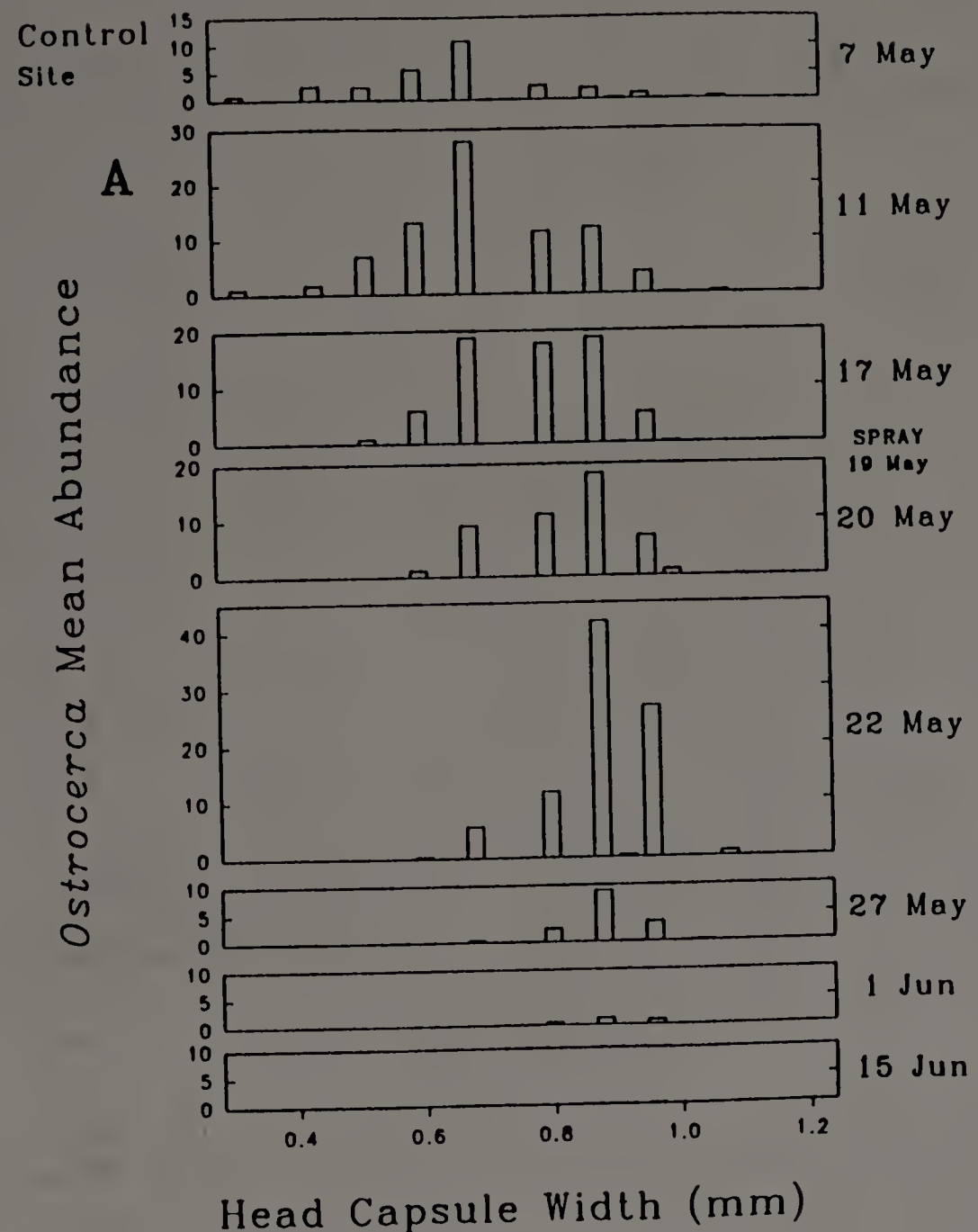

B
Control
Treated
Ostrocerca Mean Abundance
40
30
20
10
0
Spray
May
June

Fig. 17. *Ostrocerca* mean abundance in Surber samples by size class with time, control (A), treated site (B).


Control Site
A
7 May
11 May
17 May
SPRAY 10 May
20 May
22 May
27 May
1 Jun
15 Jun
Ostrocerca Mean Abundance
Head Capsule Width (mm)

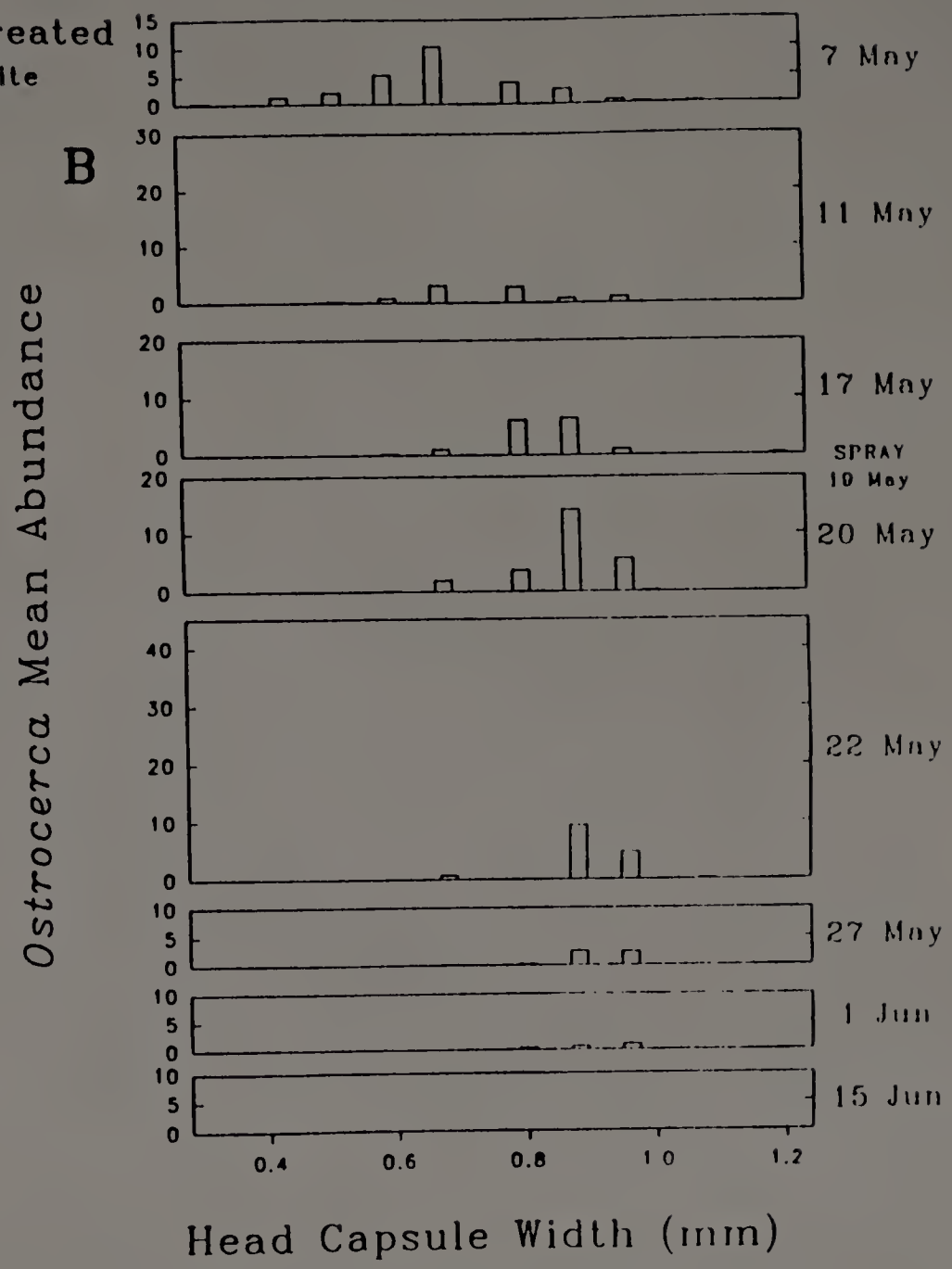

Treated Site
B
7 May
11 May
17 May
SPRAY 10 May
20 May
22 May
27 May
1 Jun
15 Jun
Ostrocerca Mean Abundance
Head Capsule Width (mm)

Fig. 18. *Ostrocerca* mean abundance in drift samples by size class with time, control (A), treated site (B).

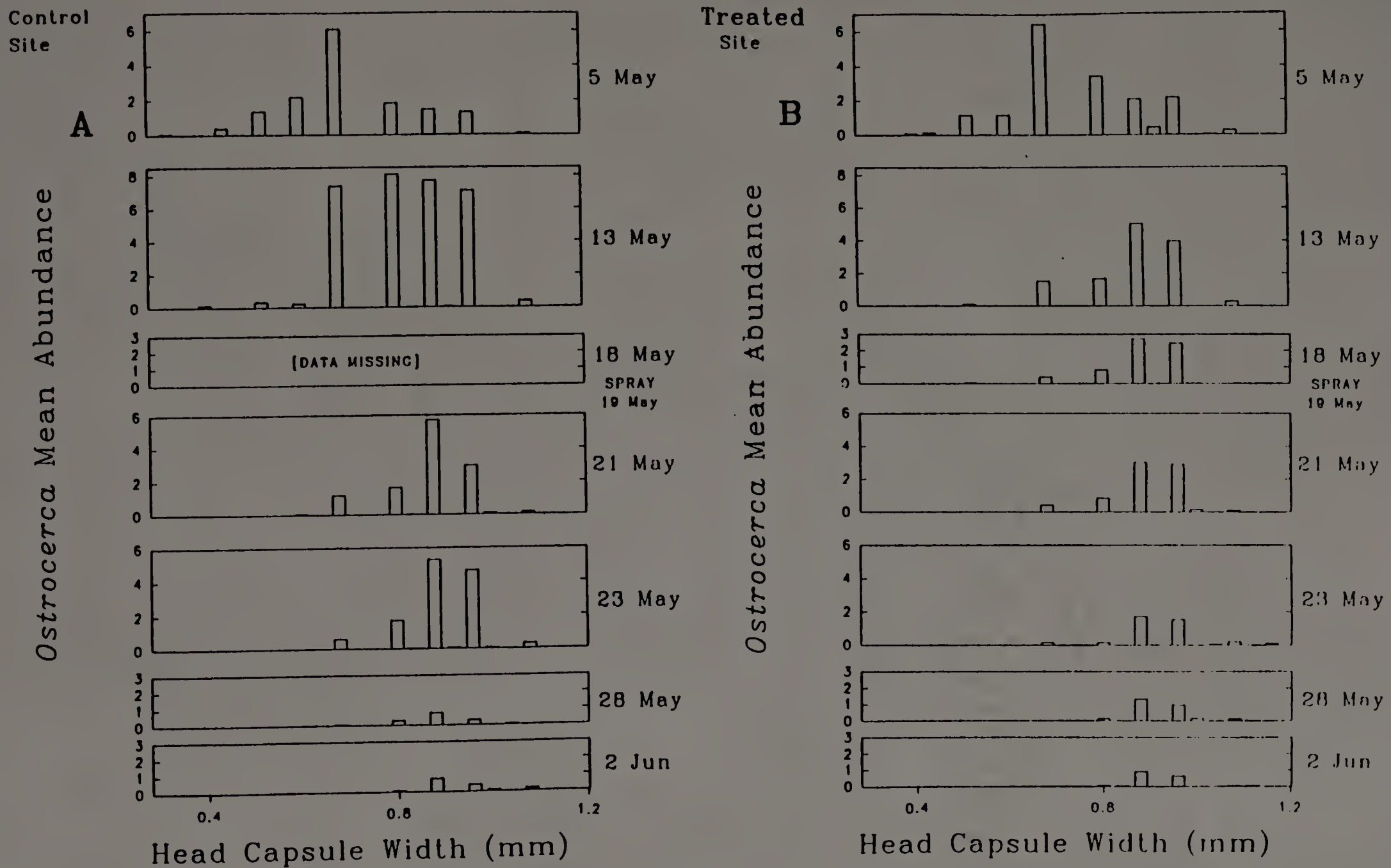

Control Site
A
Treated Site
B
Ostrocerca Mean Abundance
5 May
13 May
18 May
[DATA MISSING]
SPRAY 19 May
21 May
23 May
28 May
2 Jun
0.4
0.8
1.2
Head Capsule Width (mm)

Fig. 19. *Siphlonurus* mean abundance by site with time, ± 1 S.E., drift samples.

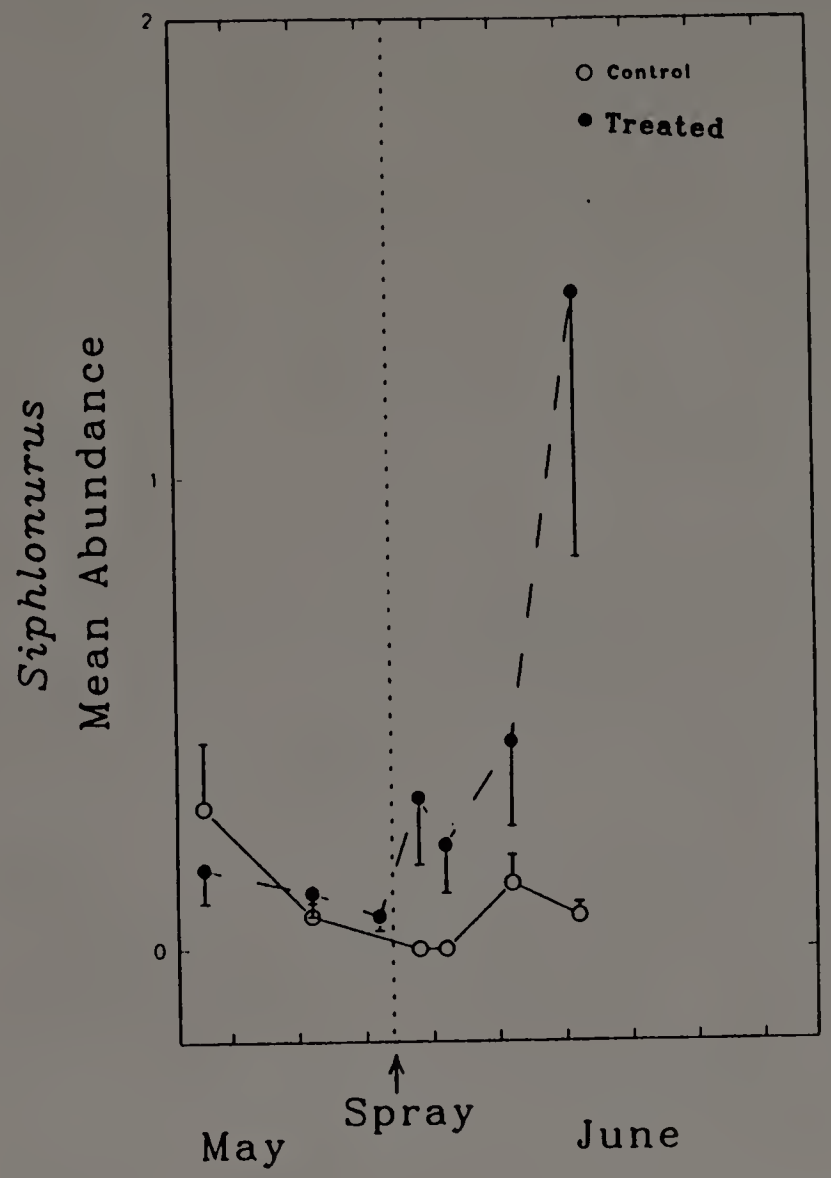
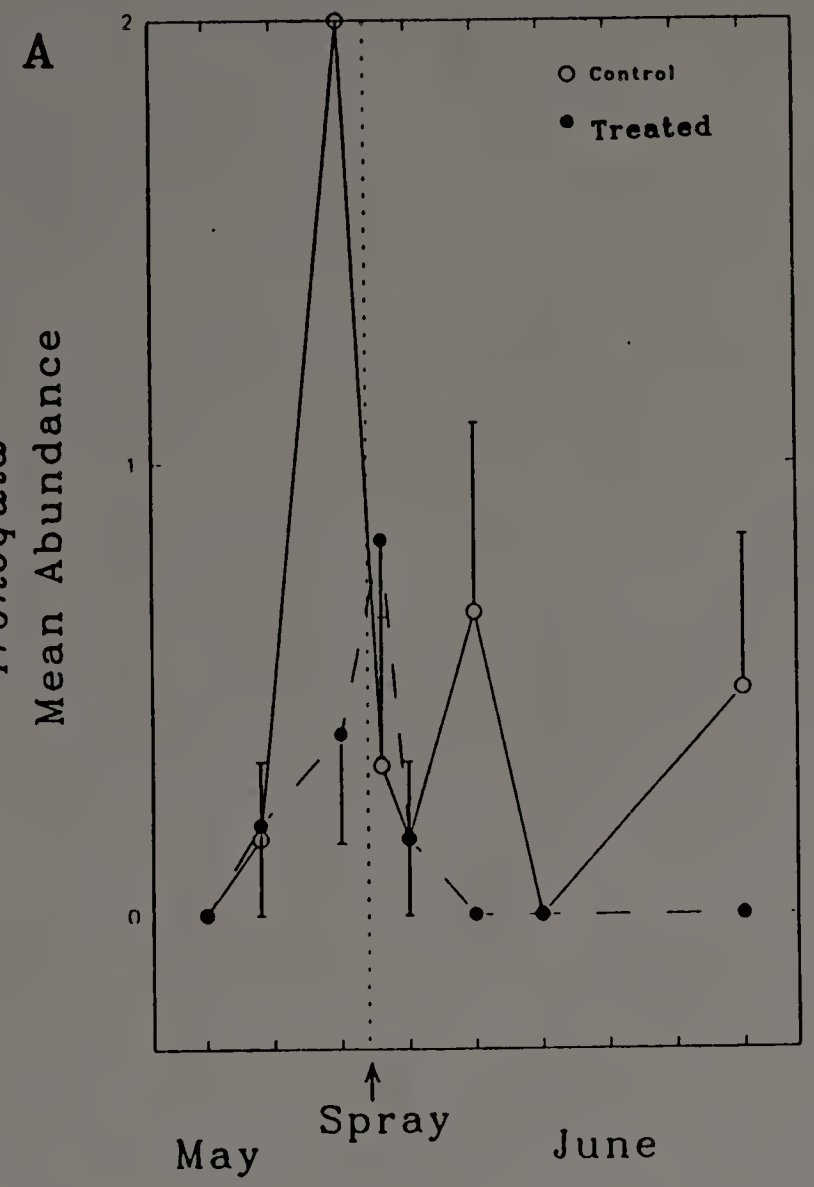

Siphlonurus
Mean Abundance
2
1
0
Control
Treated
May
Spray
June

Fig. 20. *Ironoquia* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
2
1
0
Control
Treated
Ironoquia
Mean Abundance
May
Spray
June

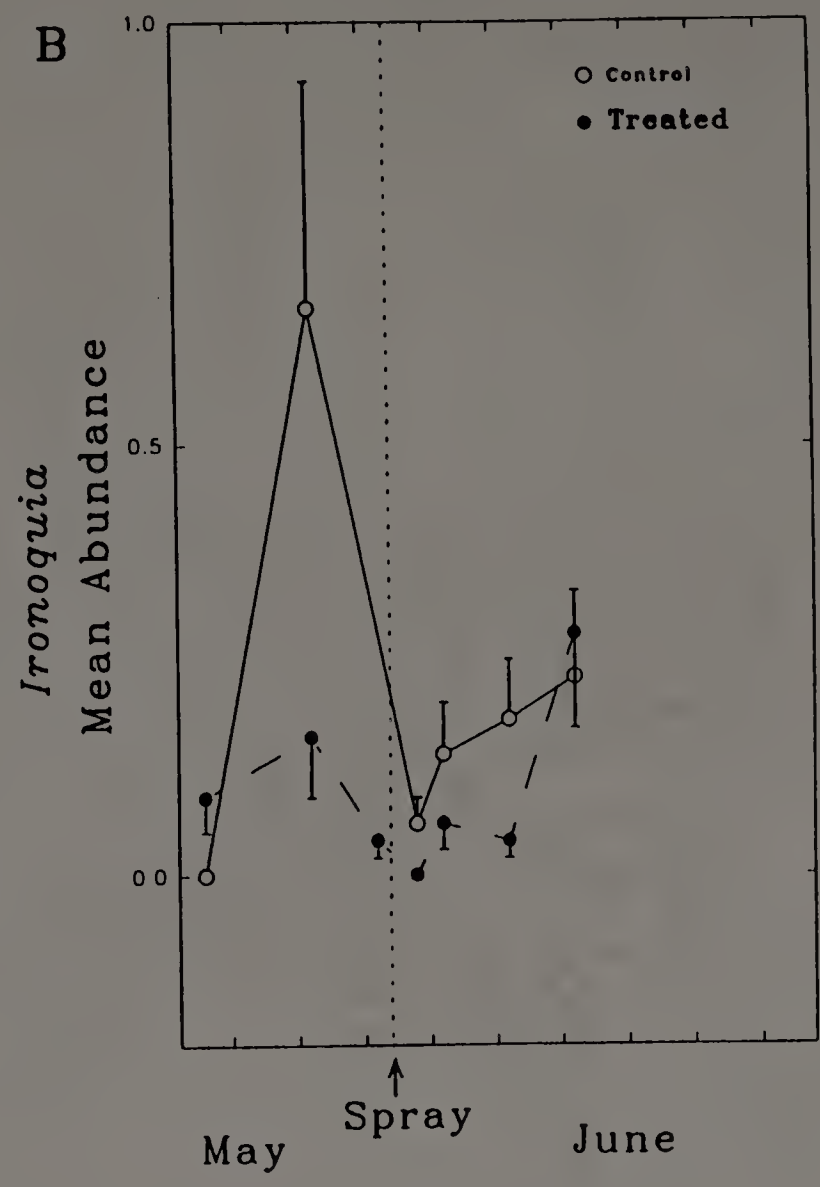
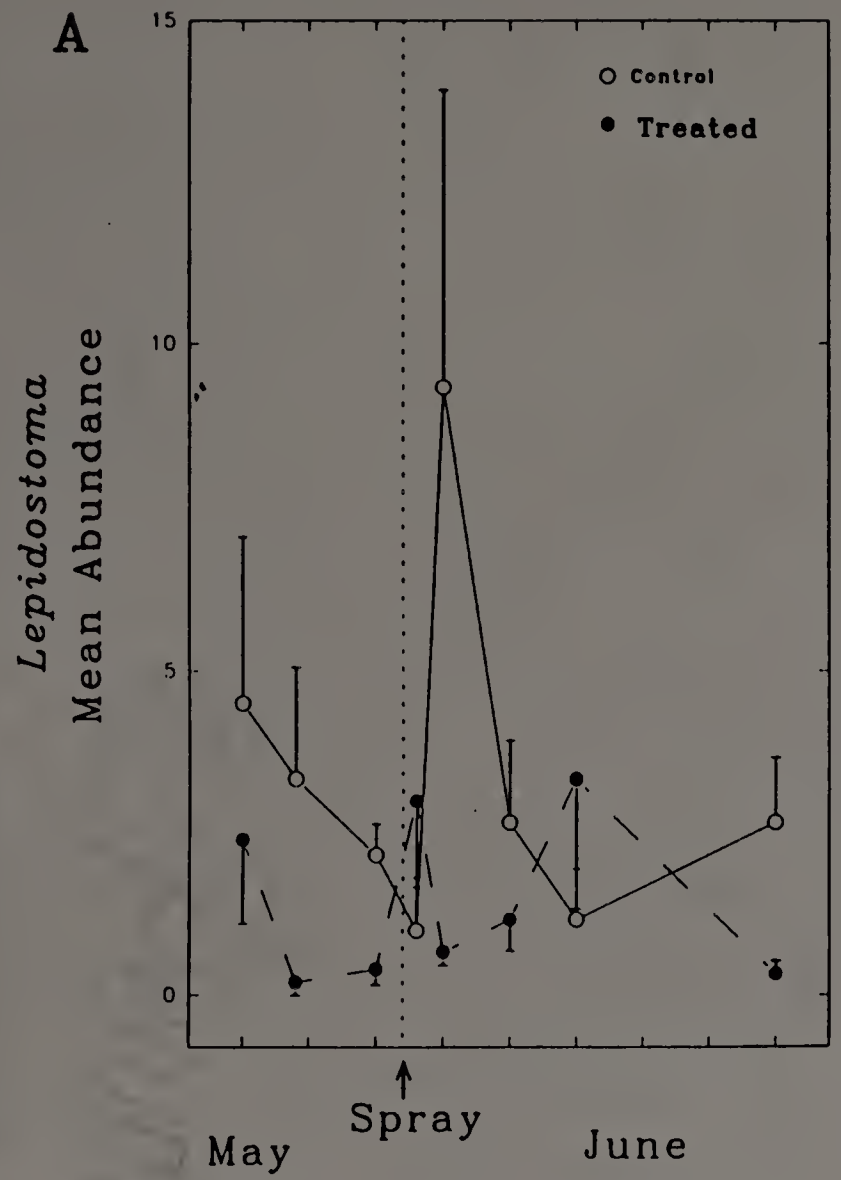

B
1.0
0.5
0 0
Control
Treated
Ironoquia
Mean Abundance
May
Spray
June

Fig. 21. *Lepidostoma* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
15
10
5
0
O Control
• Treated
Lepidostoma
Mean Abundance
May
Spray
June

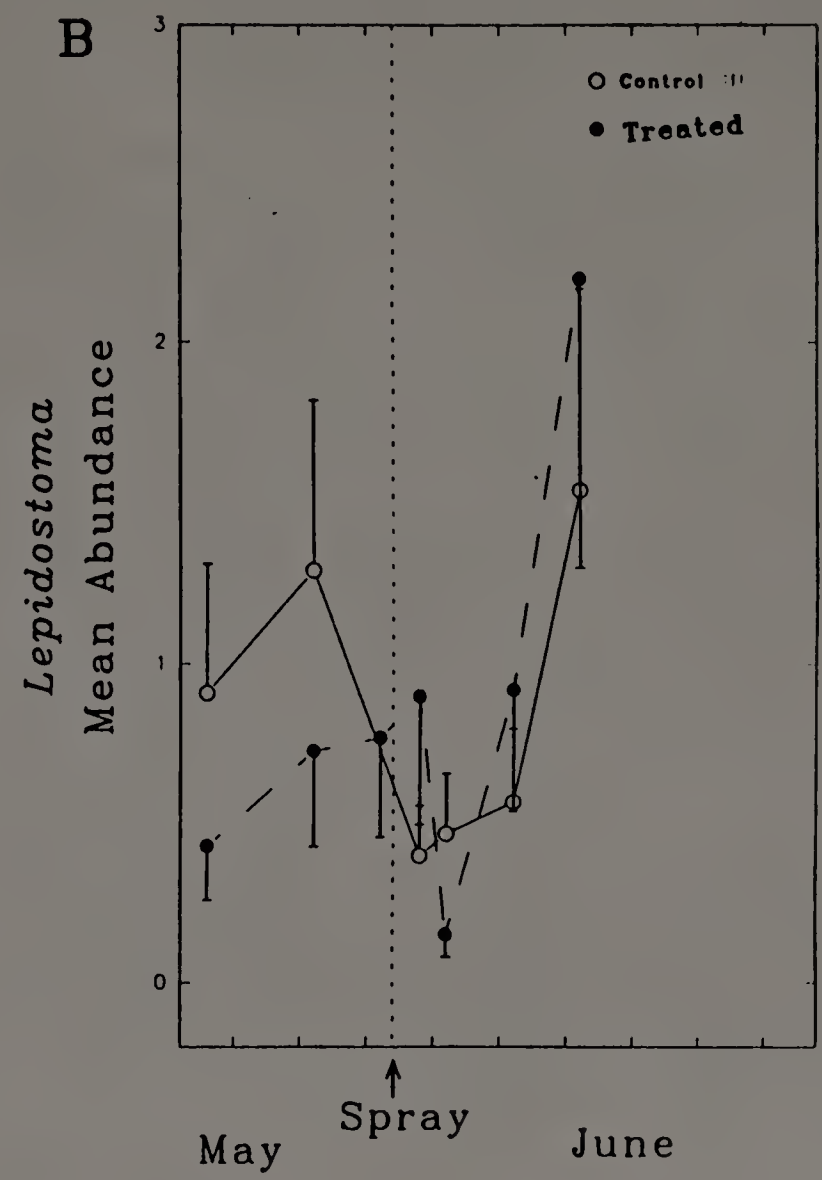
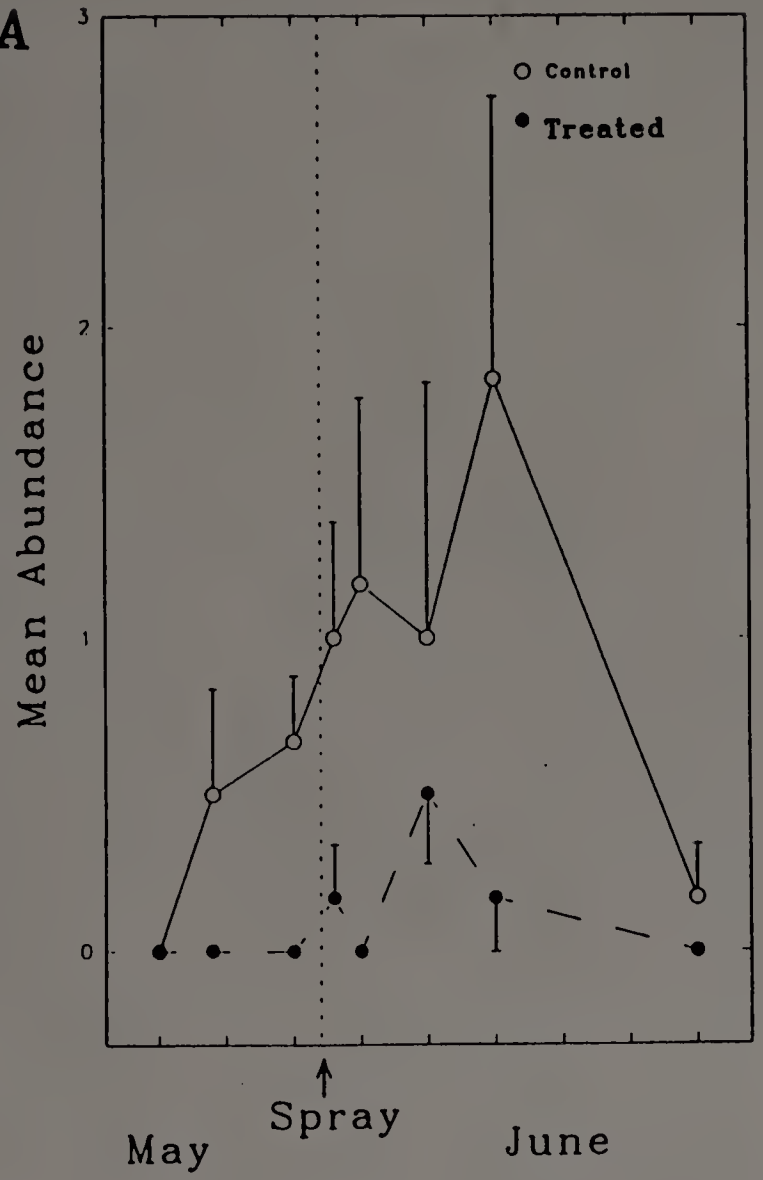

B
3
2
1
0
O Control
• Treated
Lepidostoma
Mean Abundance
May
Spray
June

Fig. 22. *Neophylax* mean abundance by site with time, ± 1 S.E., Surber samples (A), and *Palaegabus* mean abundance by site with time, Surber samples (B).


A
Neophylax
Mean Abundance
3
2
1
0
Control
Treated
May
Spray
June

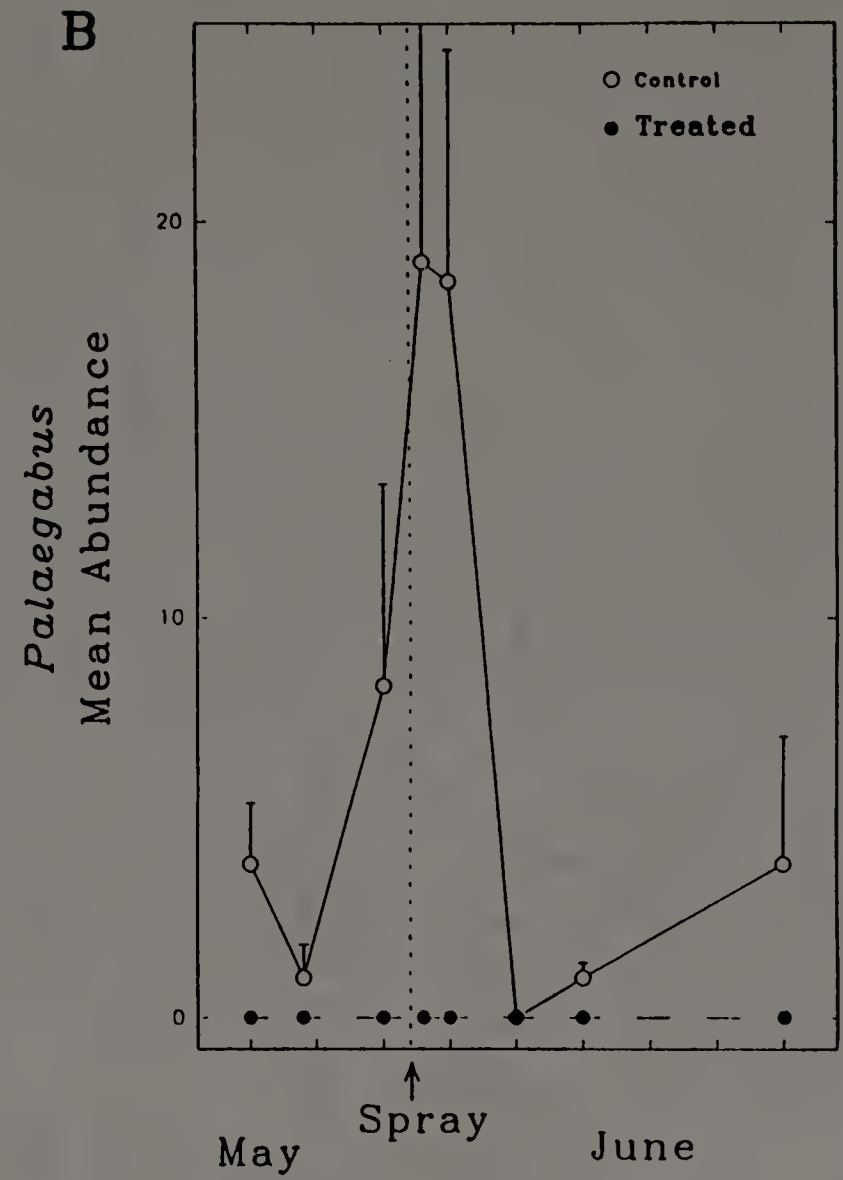
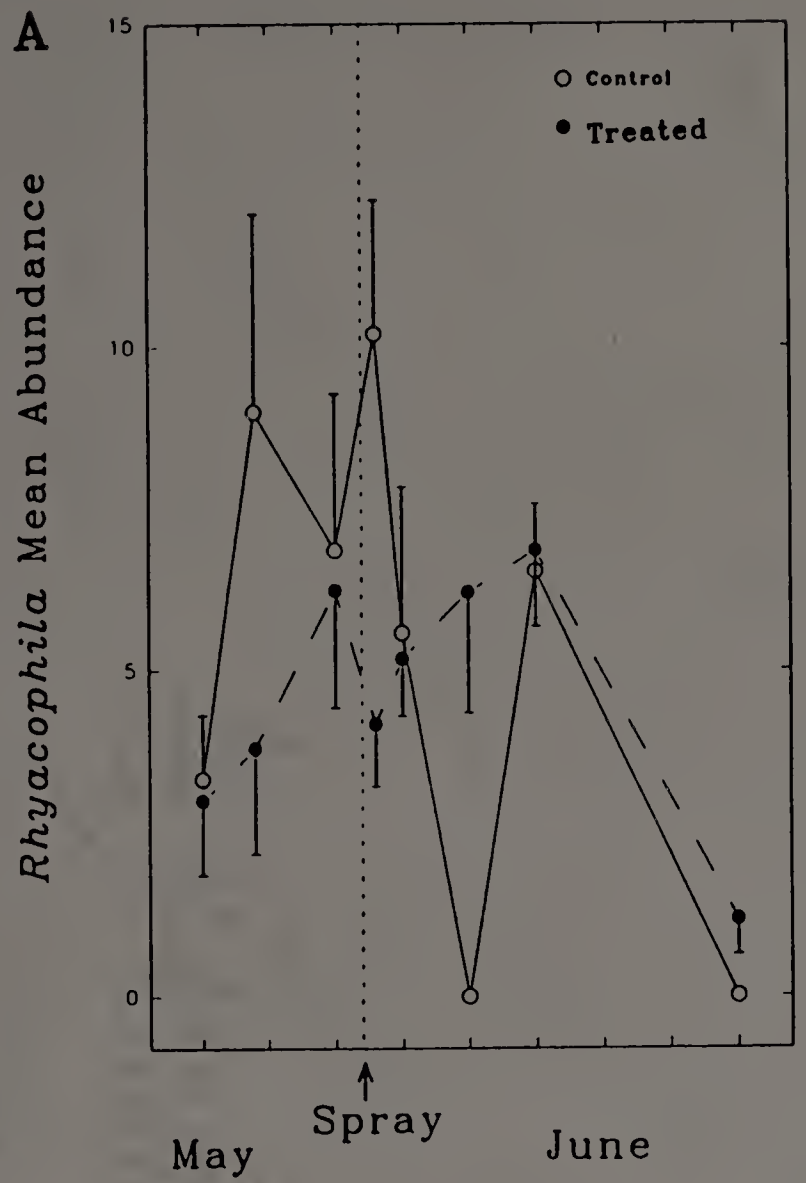

B
Palaegabus
Mean Abundance
20
10
0
Control
Treated
May
Spray
June

Fig. 23. *Rhyacophila* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
15
10
5
0
Rhyacophila Mean Abundance
Control
Treated
May
Spray
June

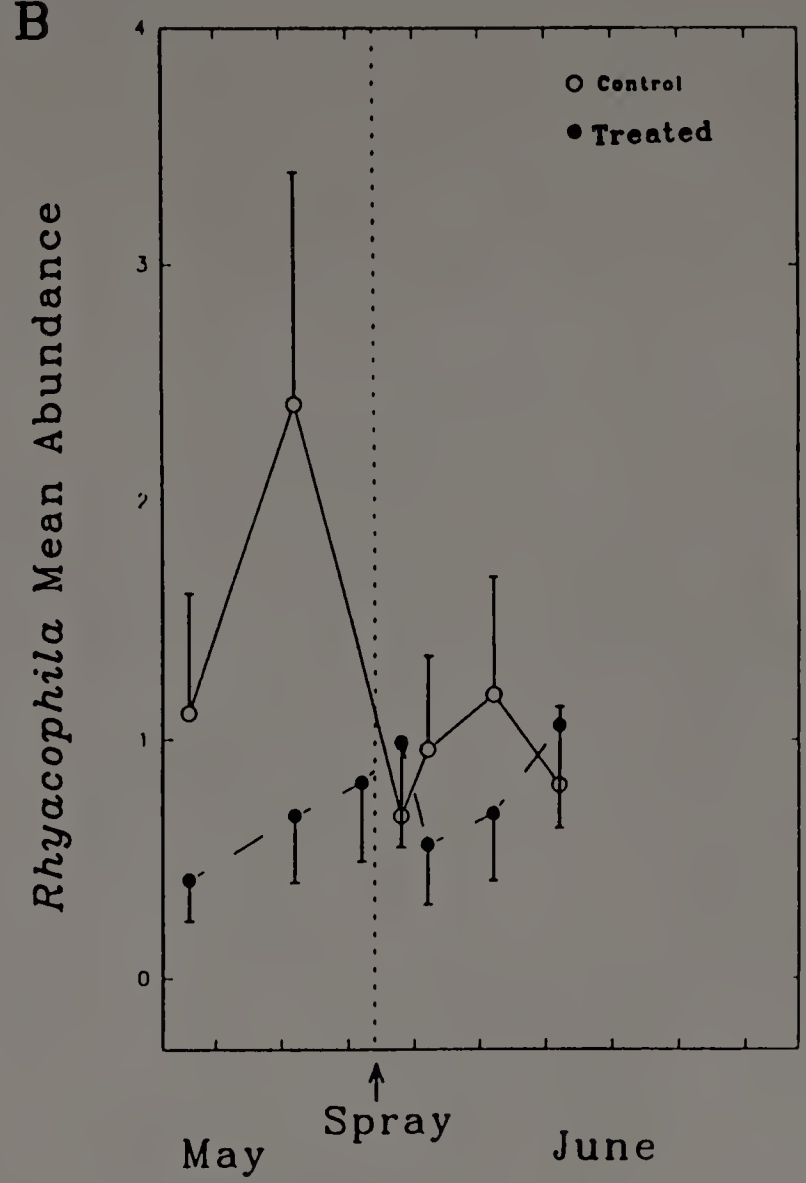
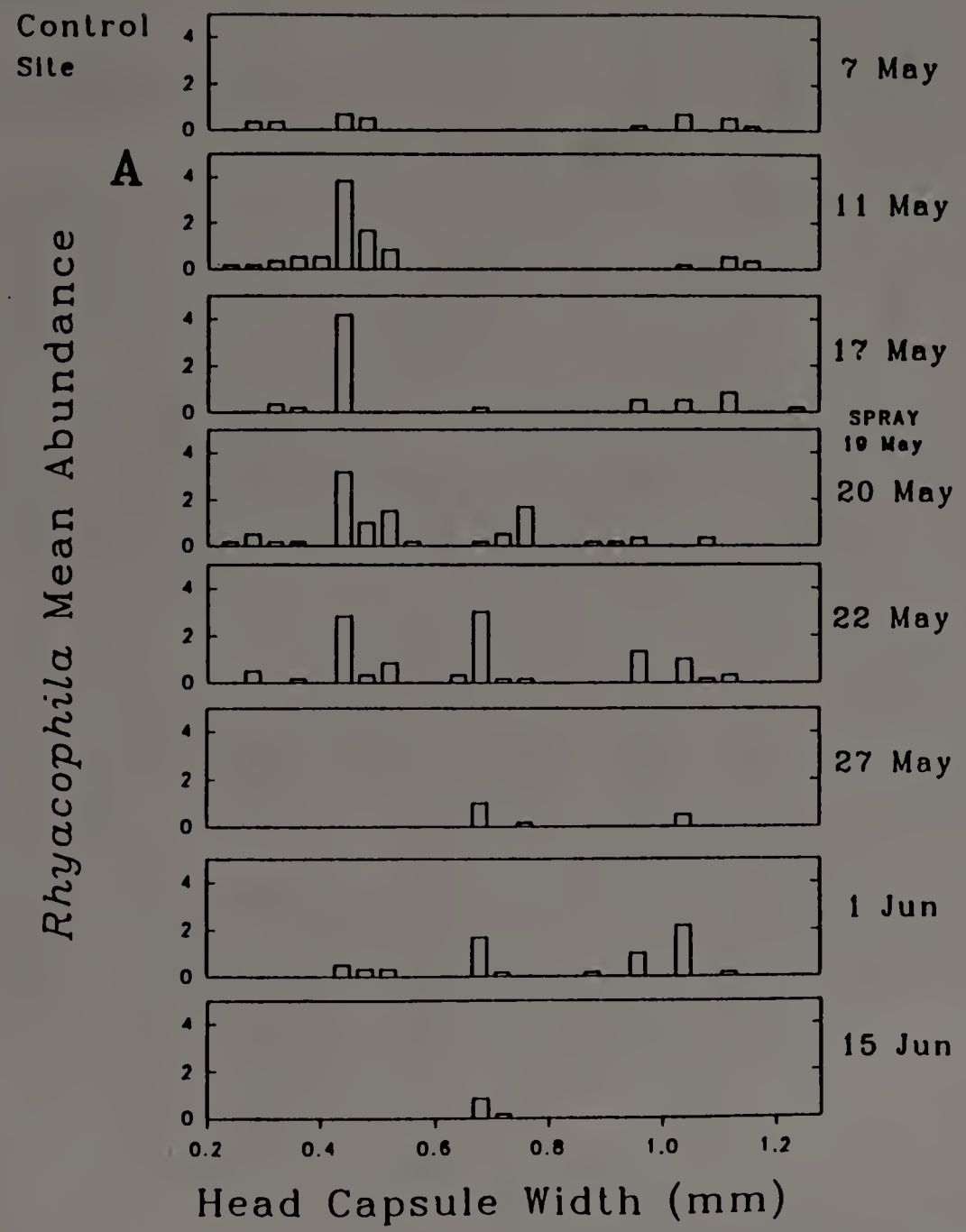

B
4
3
2
1
0
Rhyacophila Mean Abundance
Control
Treated
May
Spray
June

Fig. 24. *Rhyacophila* mean abundance in Surber samples by size class with time, control (A), treated site (B).


Control
Site
A
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
Rhyacophila Mean Abundance
Head Capsule Width (mm)

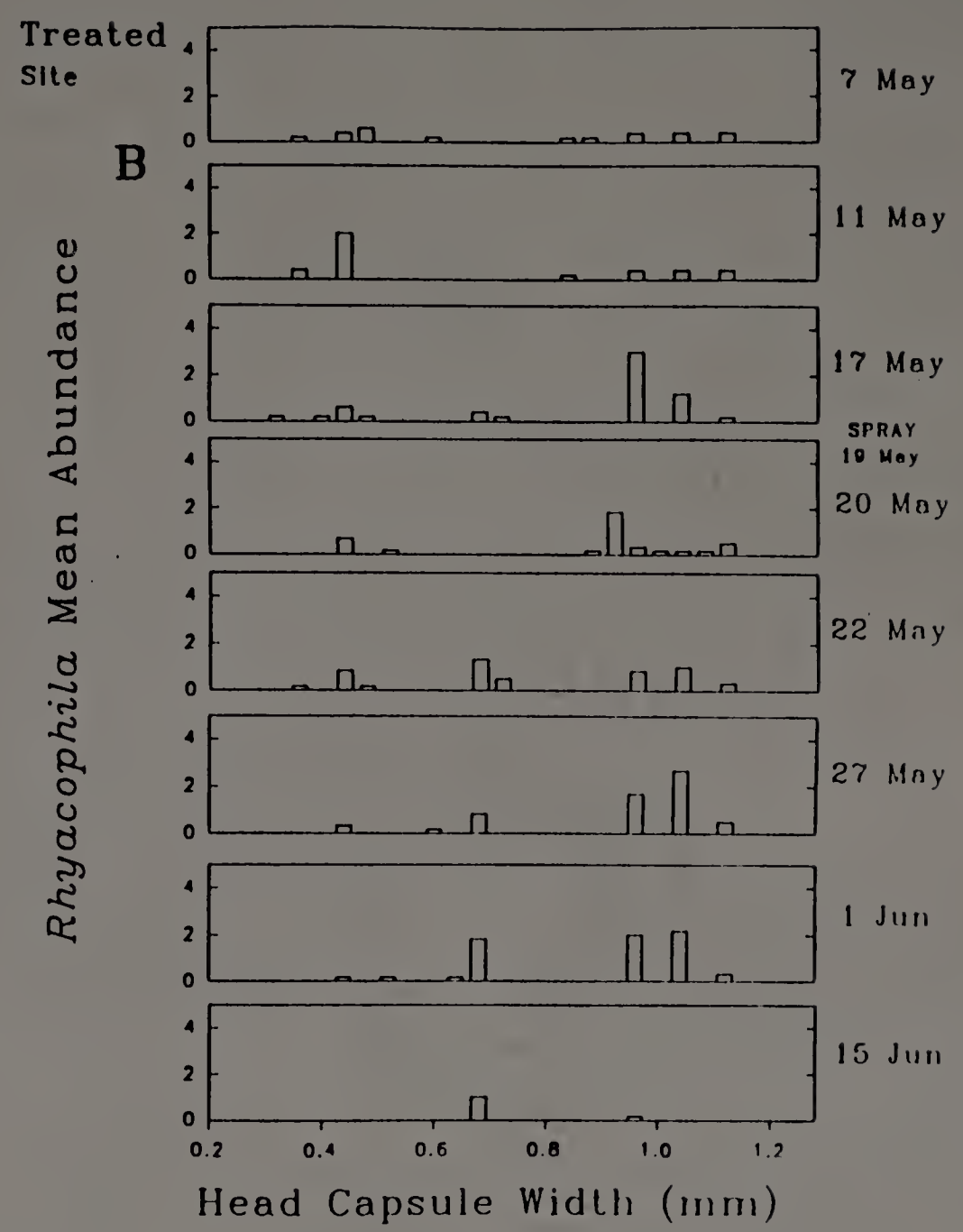
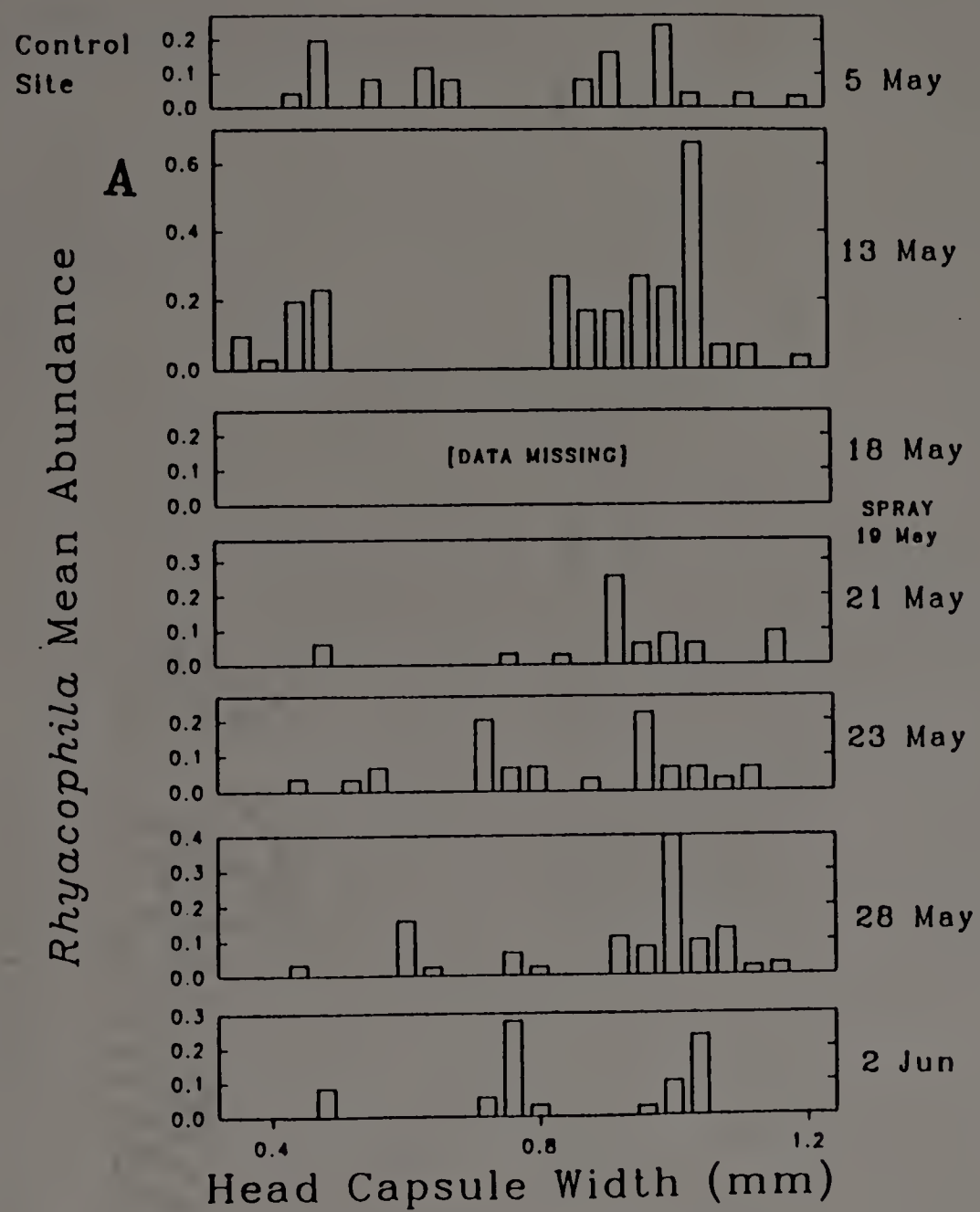

Treated
Site
B
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
Rhyacophila Mean Abundance
Head Capsule Width (mm)

Fig. 25. *Rhyacophila* mean abundance in drift samples by size class with time, control (A), treated site (B).


Control Site
A
5 May
13 May
[DATA MISSING]
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Rhyacophila Mean Abundance
Head Capsule Width (mm)

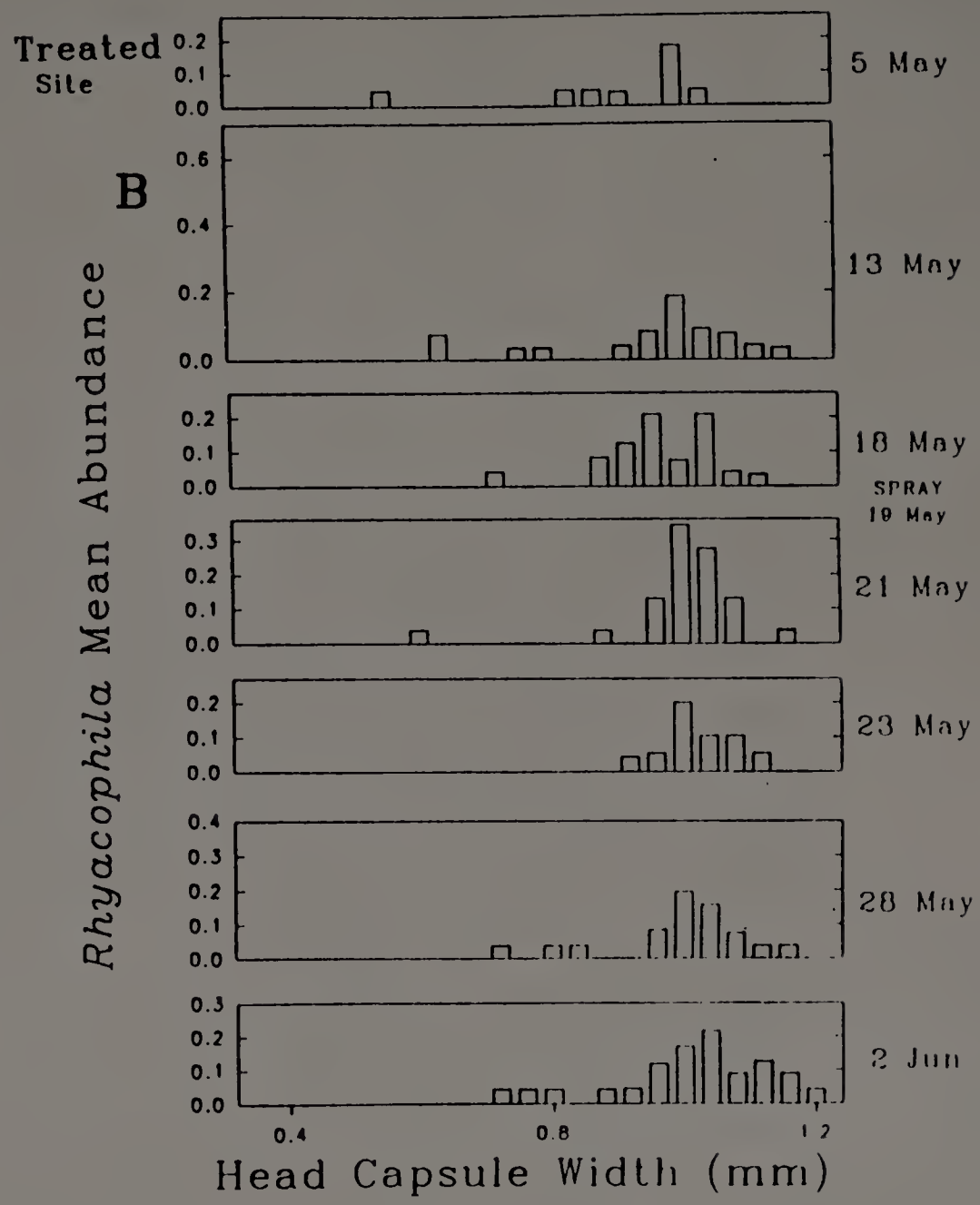
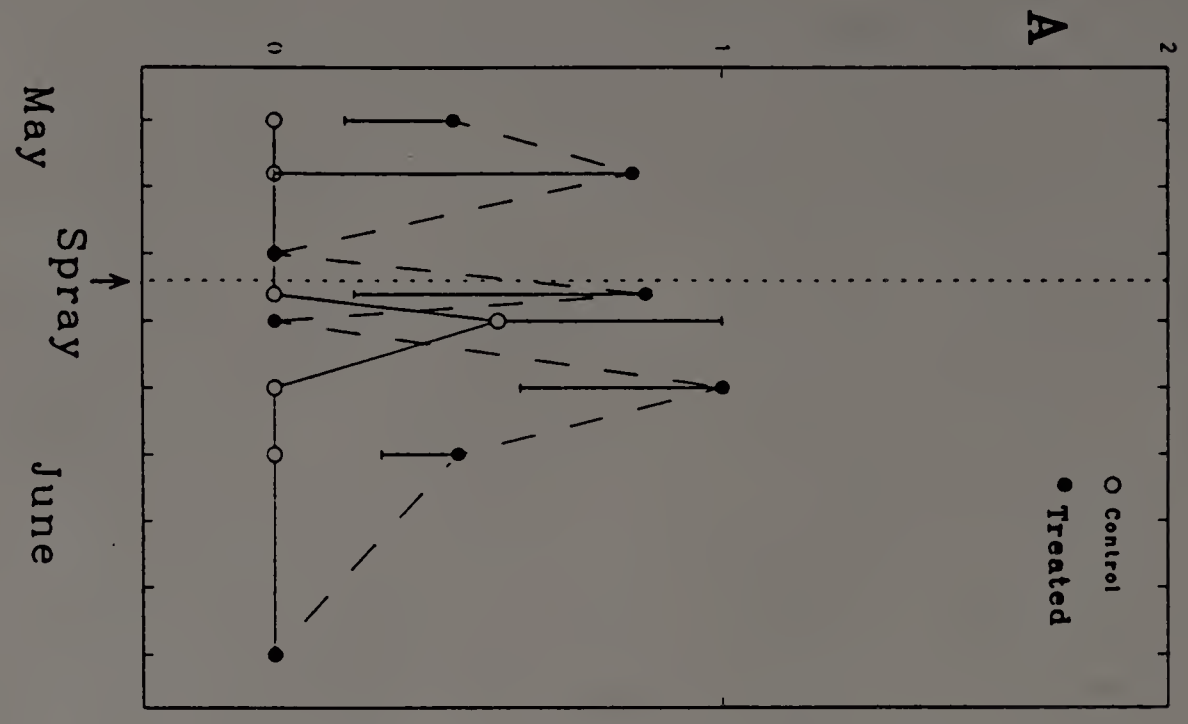

Treated Site
B
5 May
13 May
18 May
SPRAY 19 May
21 May
23 May
28 May
2 Jun
Rhyacophila Mean Abundance
Head Capsule Width (mm)

Fig. 26. *Prosimulium magnum* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).

Prosimulium magnum complex Mean Abundance



Prosimulium magnum complex

Mean Abundance

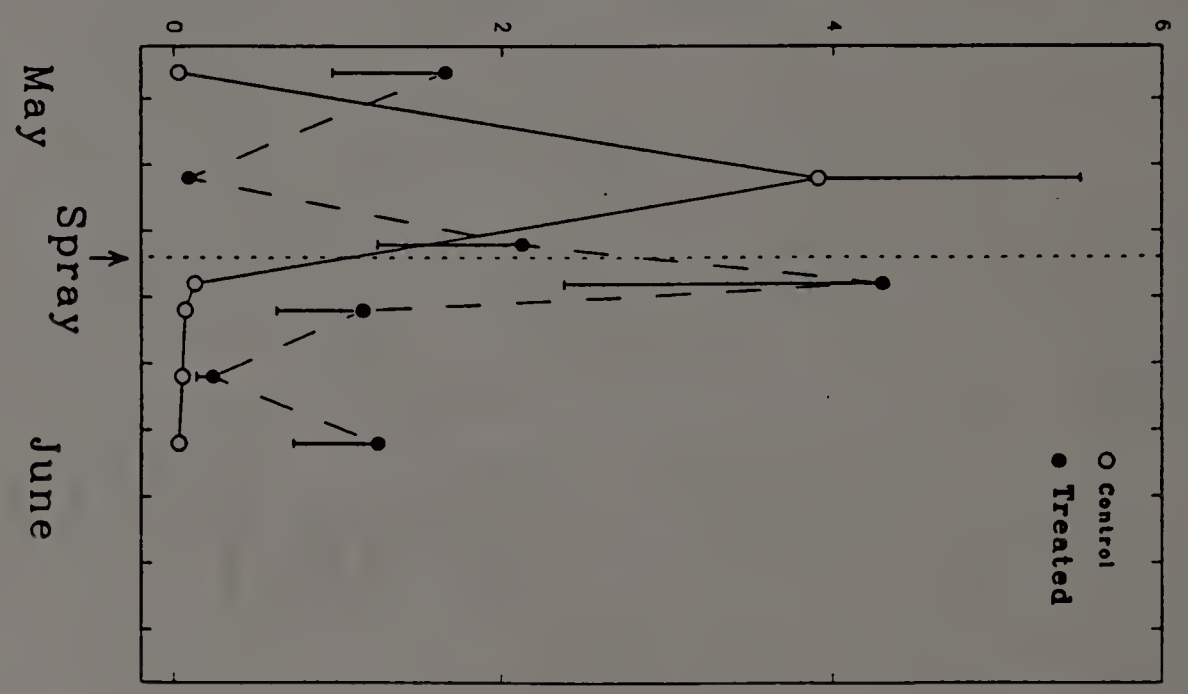
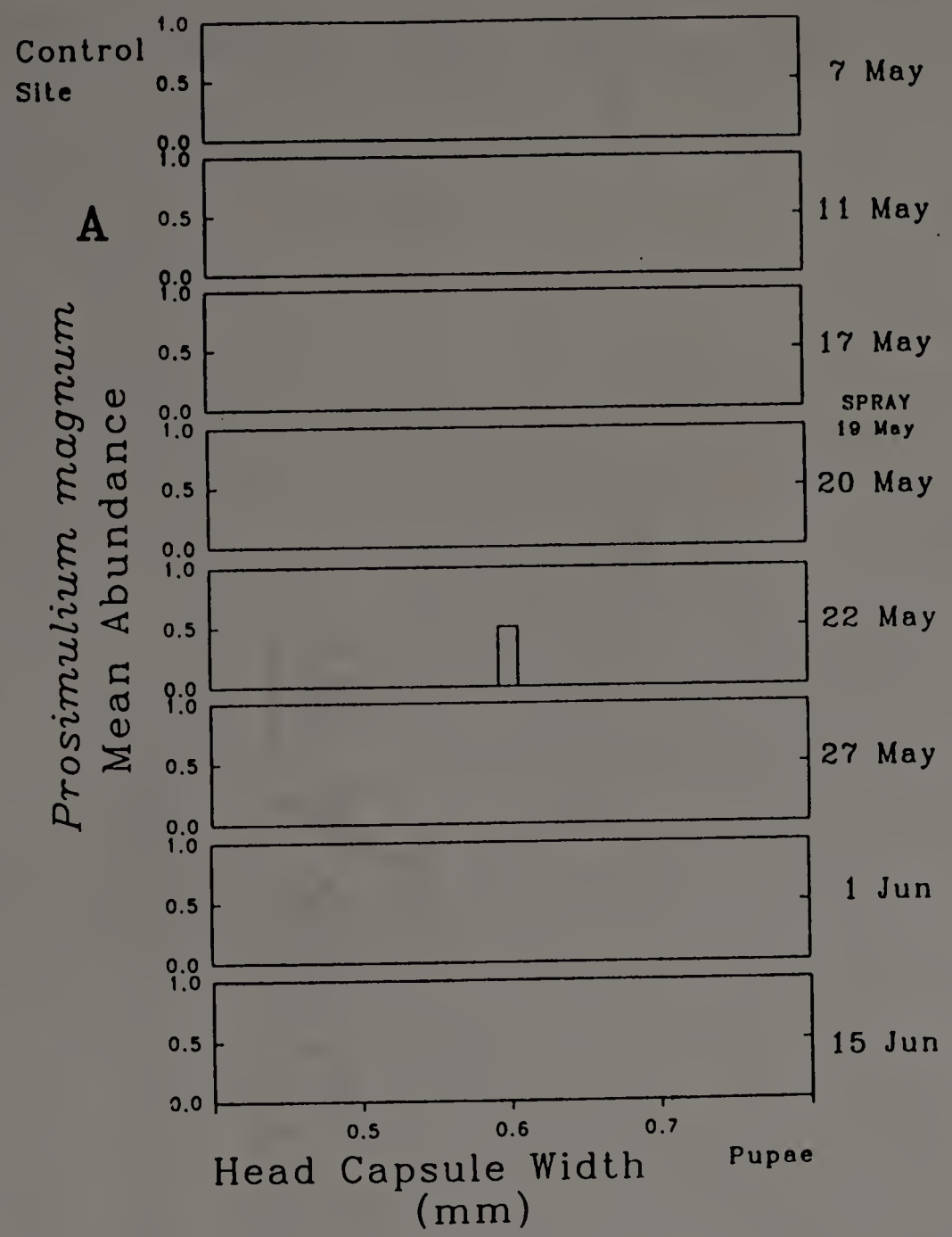


Fig. 27. *Prosimulium magnum* mean abundance in Surber samples by size class with time, larvae (open bars), pupae (hatched bars), control site (A), treated site (B).


Control
Site
A
Prosimulium magnum
Mean Abundance
1.0
0.5
0.0
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.5
0.6
0.7
Pupae
Head Capsule Width
(mm)

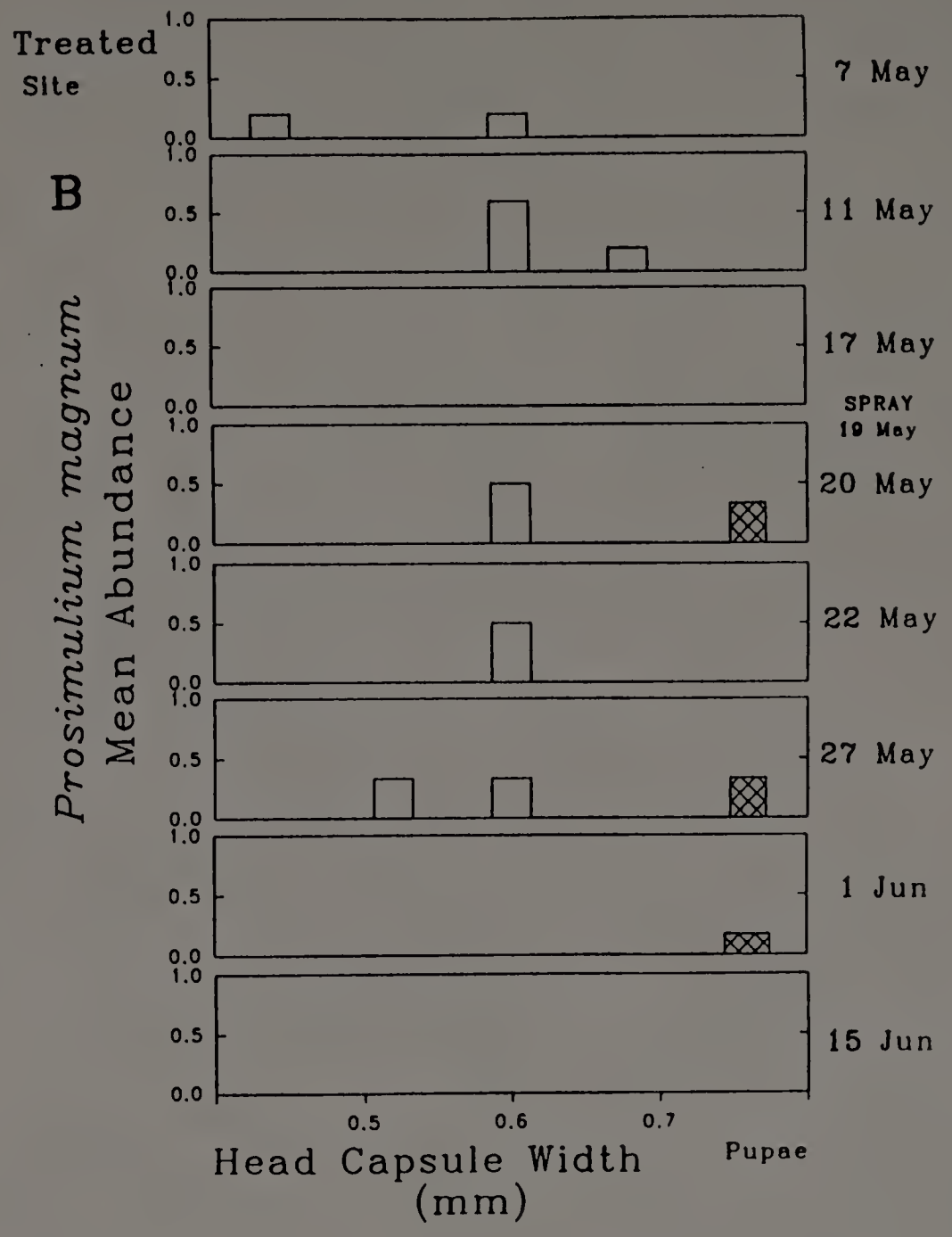
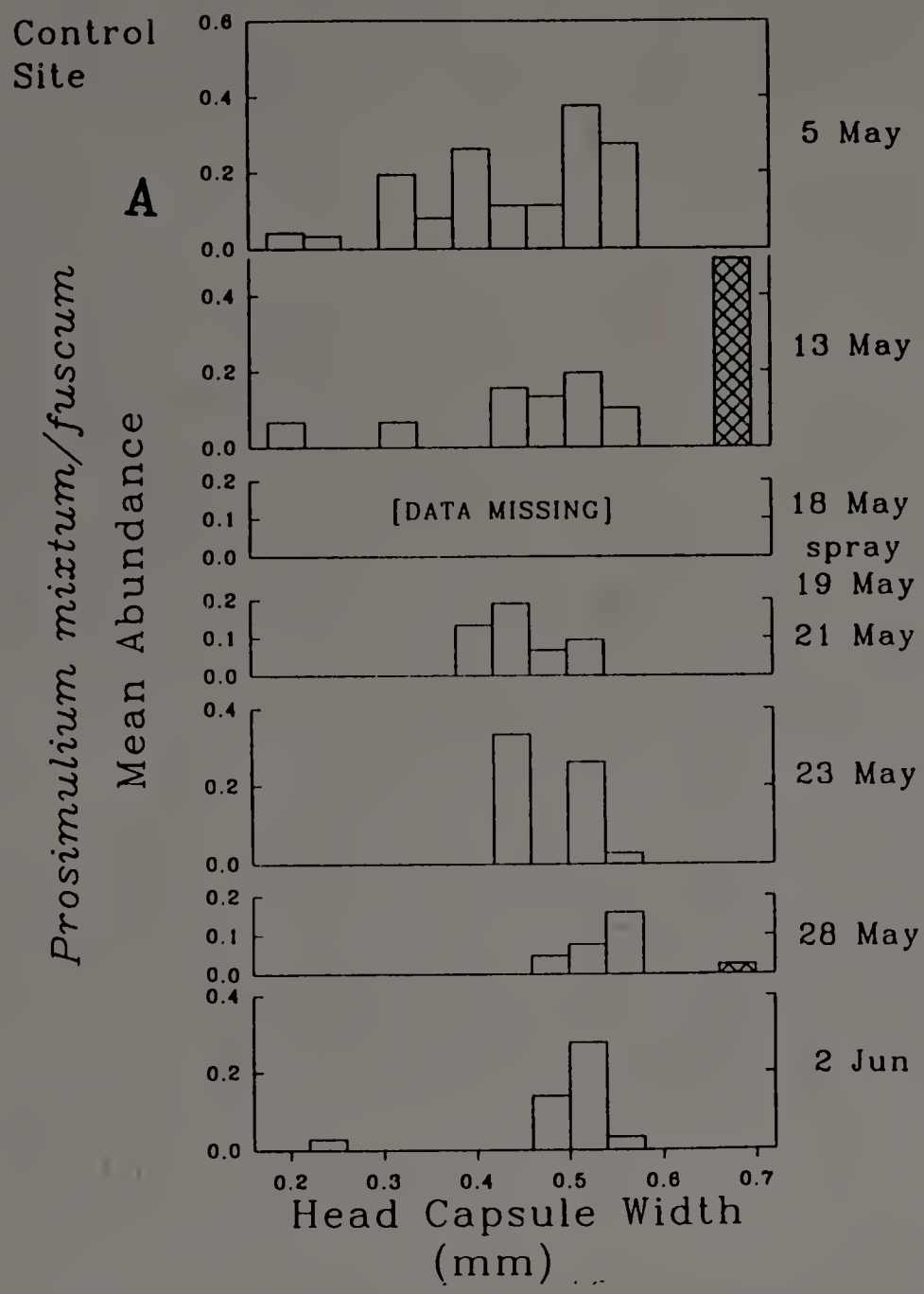

Treated
Site
B
Prosimulium magnum
Mean Abundance
1.0
0.5
0.0
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.5
0.6
0.7
Pupae
Head Capsule Width
(mm)

Fig. 28. *Prosimulium mixtum/fuscum* mean abundance in Surber samples by size class with time, larvae (open bars), pupae (hatched bars), control site (A), treated site (B).


Control Site
A
Prosimulium mixtum/fuscum
Mean Abundance
0.6
0.4
0.2
0.0
5 May
13 May
[DATA MISSING]
18 May
spray
19 May
21 May
23 May
28 May
2 Jun
0.2
0.3
0.4
0.5
0.6
0.7
Head Capsule Width
(mm)

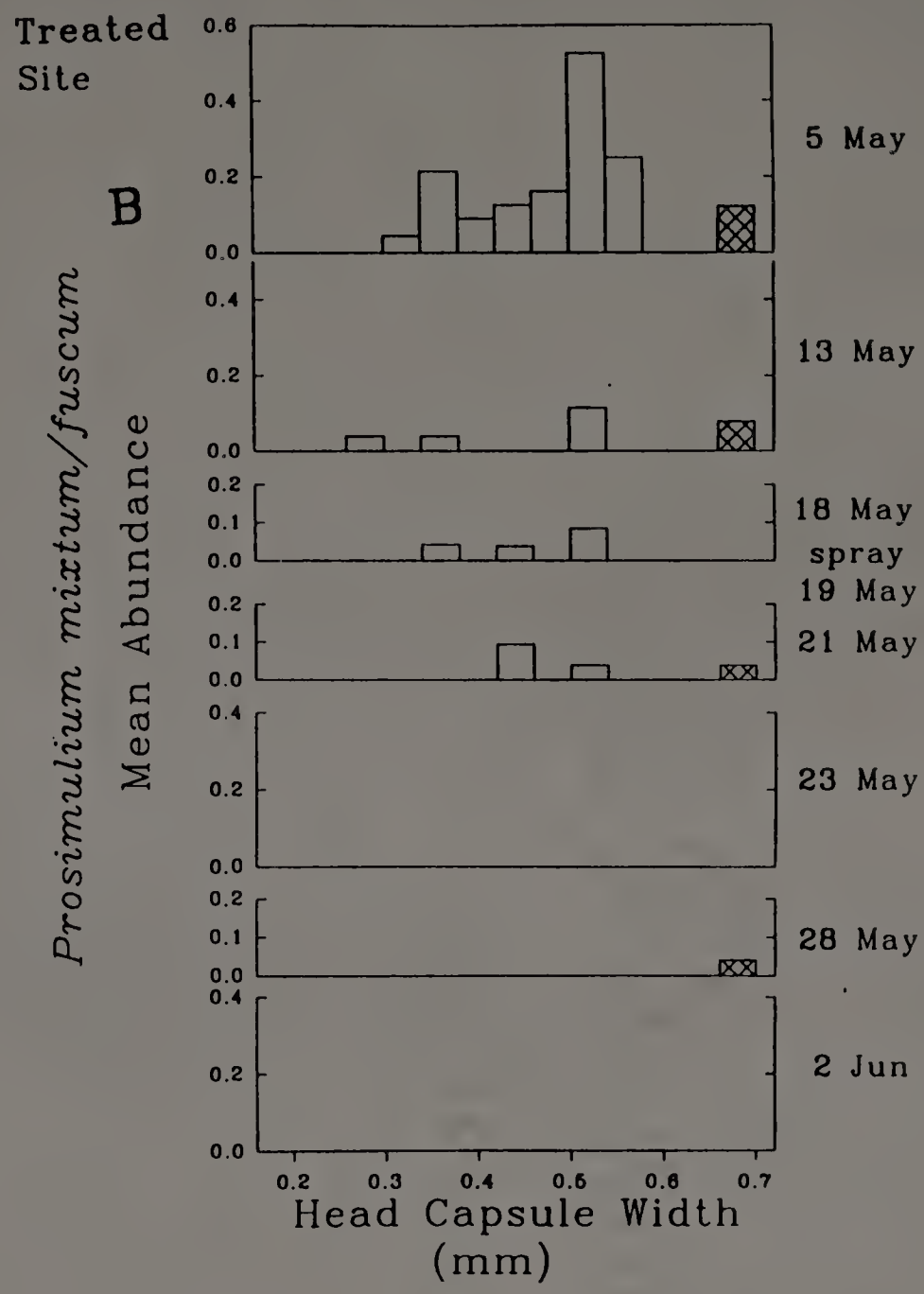
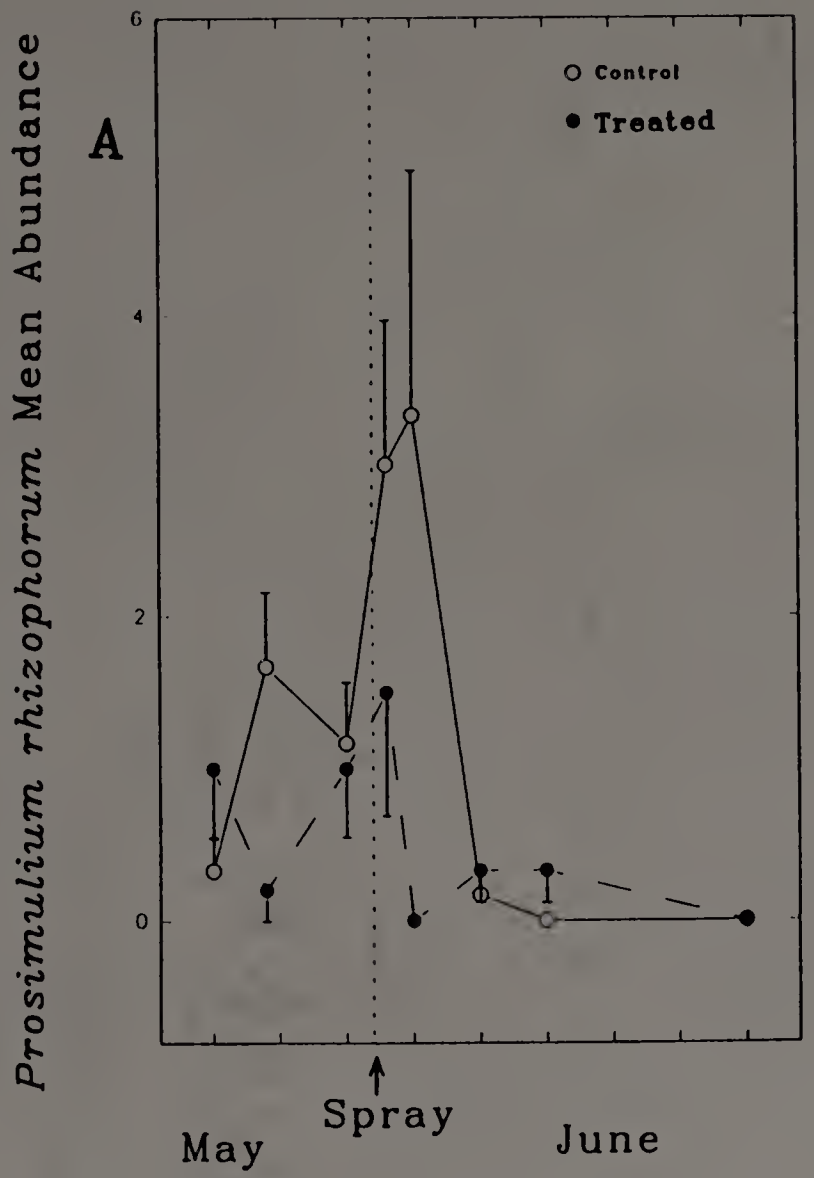

Treated Site
B
Prosimulium mixtum/fuscum
Mean Abundance
0.6
0.4
0.2
0.0
5 May
13 May
18 May
spray
19 May
21 May
23 May
28 May
2 Jun
0.2
0.3
0.4
0.5
0.6
0.7
Head Capsule Width
(mm)

Fig. 29. *Prosimulium rhizophorum* mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Prosimulium rhizophorum Mean Abundance
6
4
2
0
Control
Treated
May
Spray
June

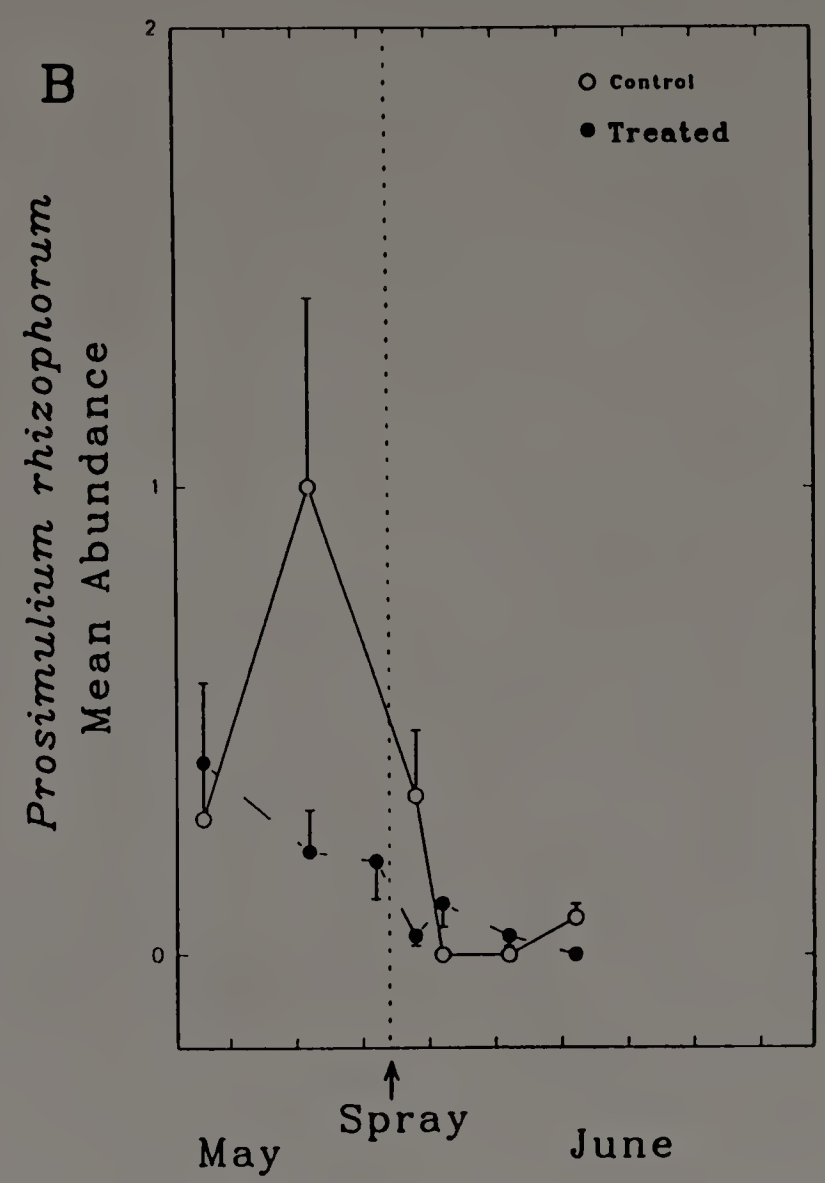
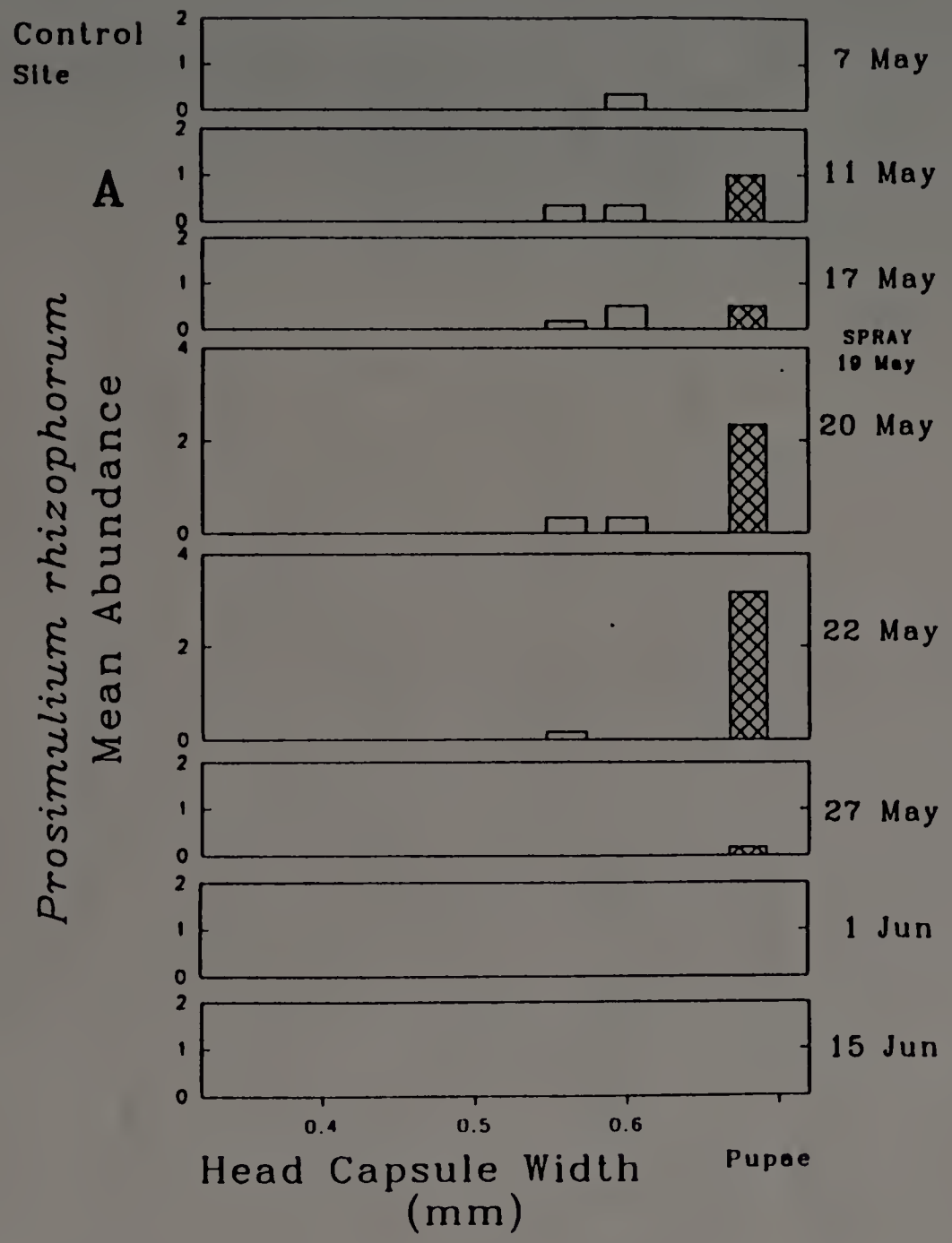

B
Prosimulium rhizophorum
Mean Abundance
2
1
0
Control
Treated
May
Spray
June

Fig. 30. *Prosimulium rhizophorum* mean abundance in Surber samples by size class with time, larvae (open bars), pupae (hatched bars), control site (A), treated site (B).


Control
Site
A
Prosimulium rhizophorum
Mean Abundance
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.4
0.5
0.6
Pupae
Head Capsule Width
(mm)

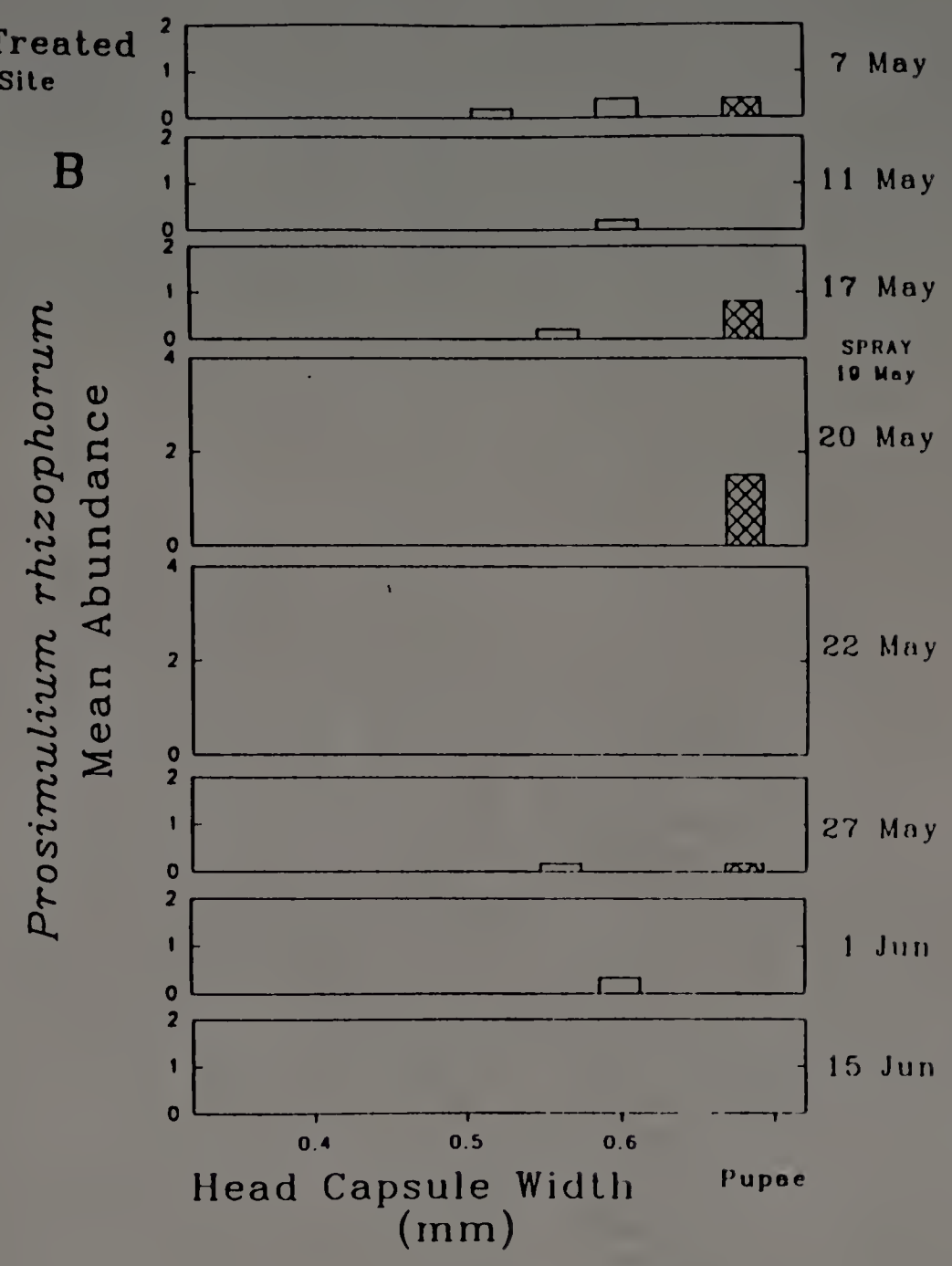
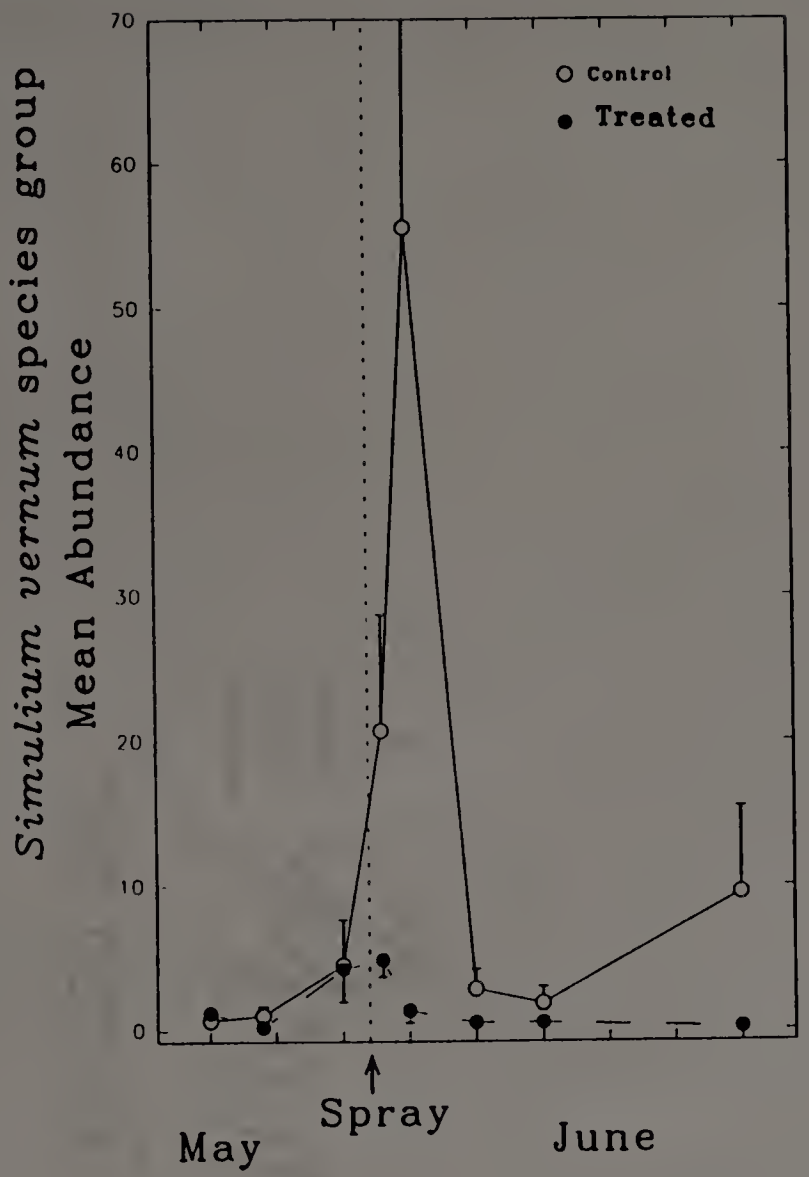

Treated
Site
B
Prosimulium rhizophorum
Mean Abundance
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.4
0.5
0.6
Pupae
Head Capsule Width
(mm)

Fig. 31. *Simulium vernum* group mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).

A


Simulium vernum species group
Mean Abundance
70
60
50
40
30
20
10
0
Control
Treated
May
Spray
June

B

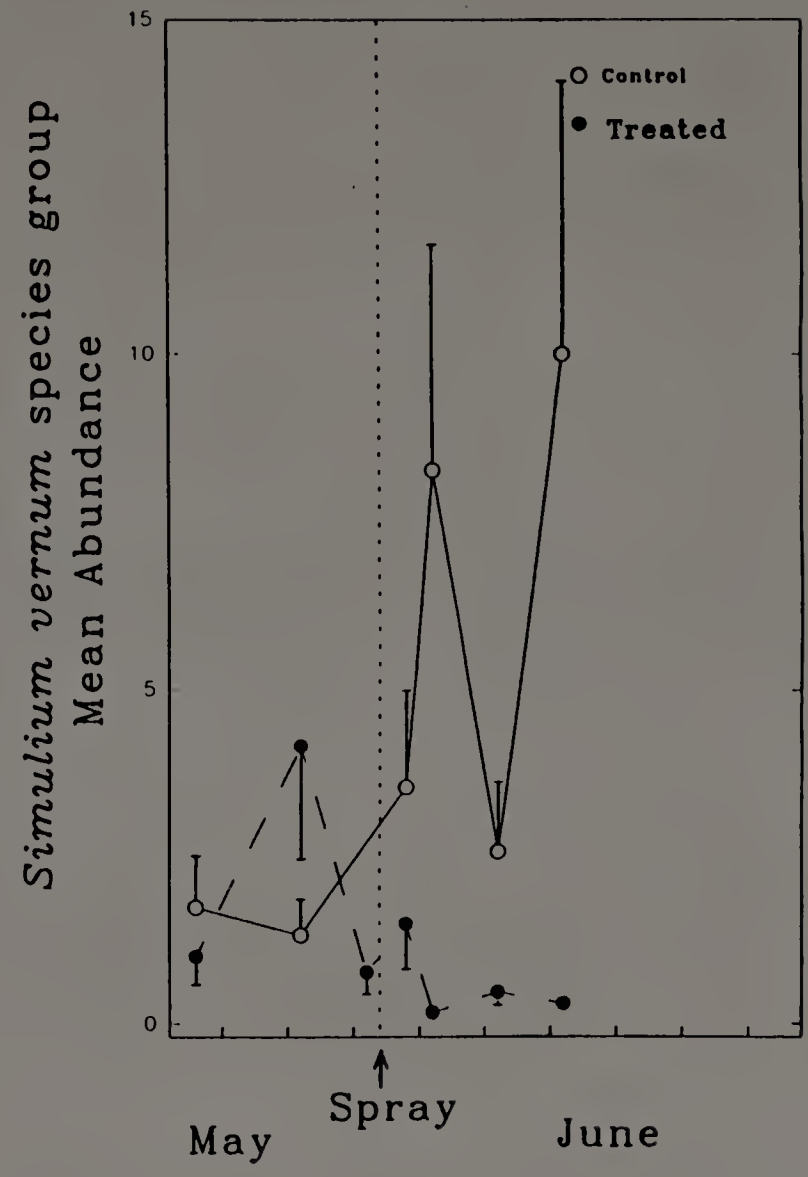
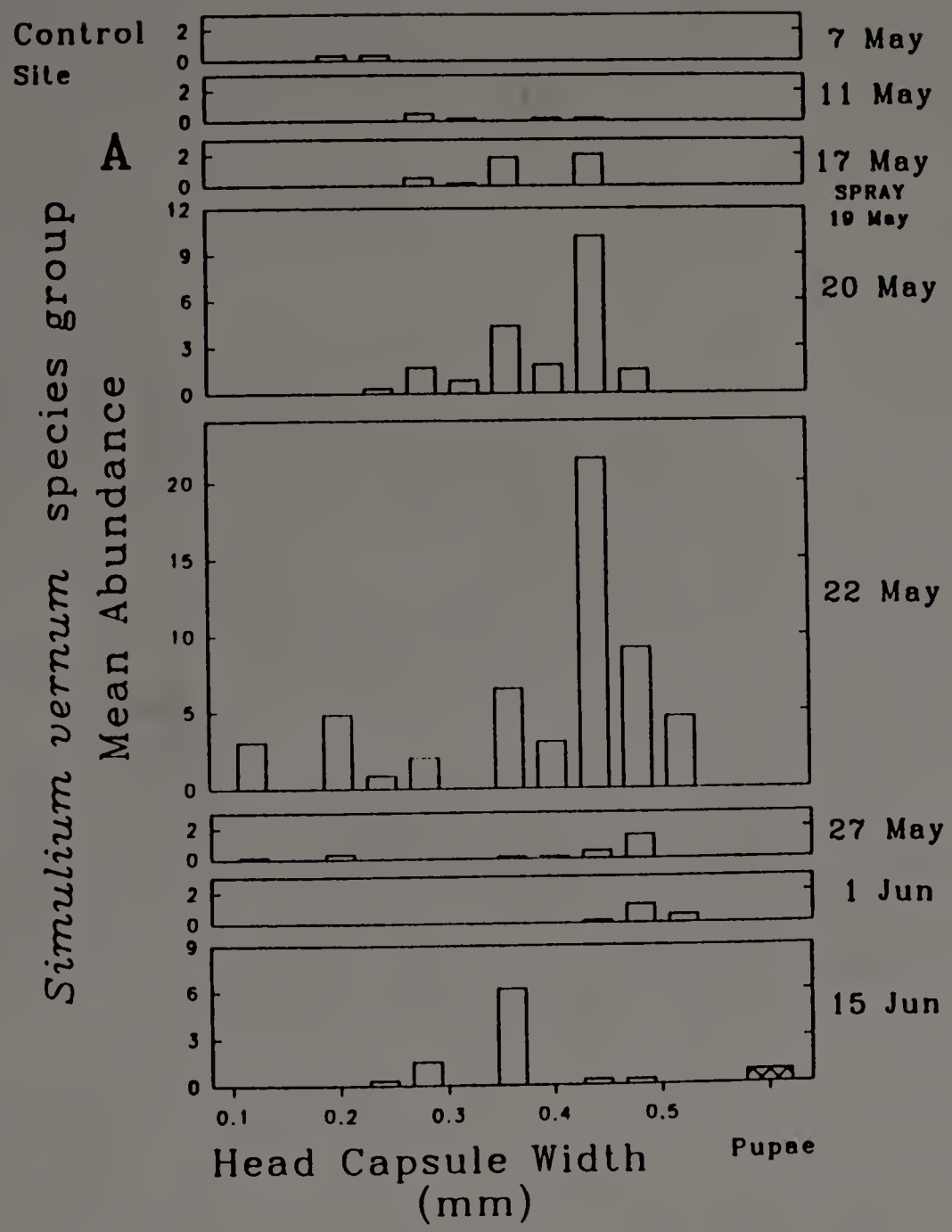

Simulium vernum species group
Mean Abundance
15
10
5
0
Control
Treated
May
Spray
June

Fig. 32. *Simulium vernum* group mean abundance in Surber samples by size class with time, larvae (open bars), pupae (hatched bars), control site (A), treated site (B).


Control
Site
A
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
Simulium vernum species group
Mean Abundance
Head Capsule Width
(mm)
Pupae

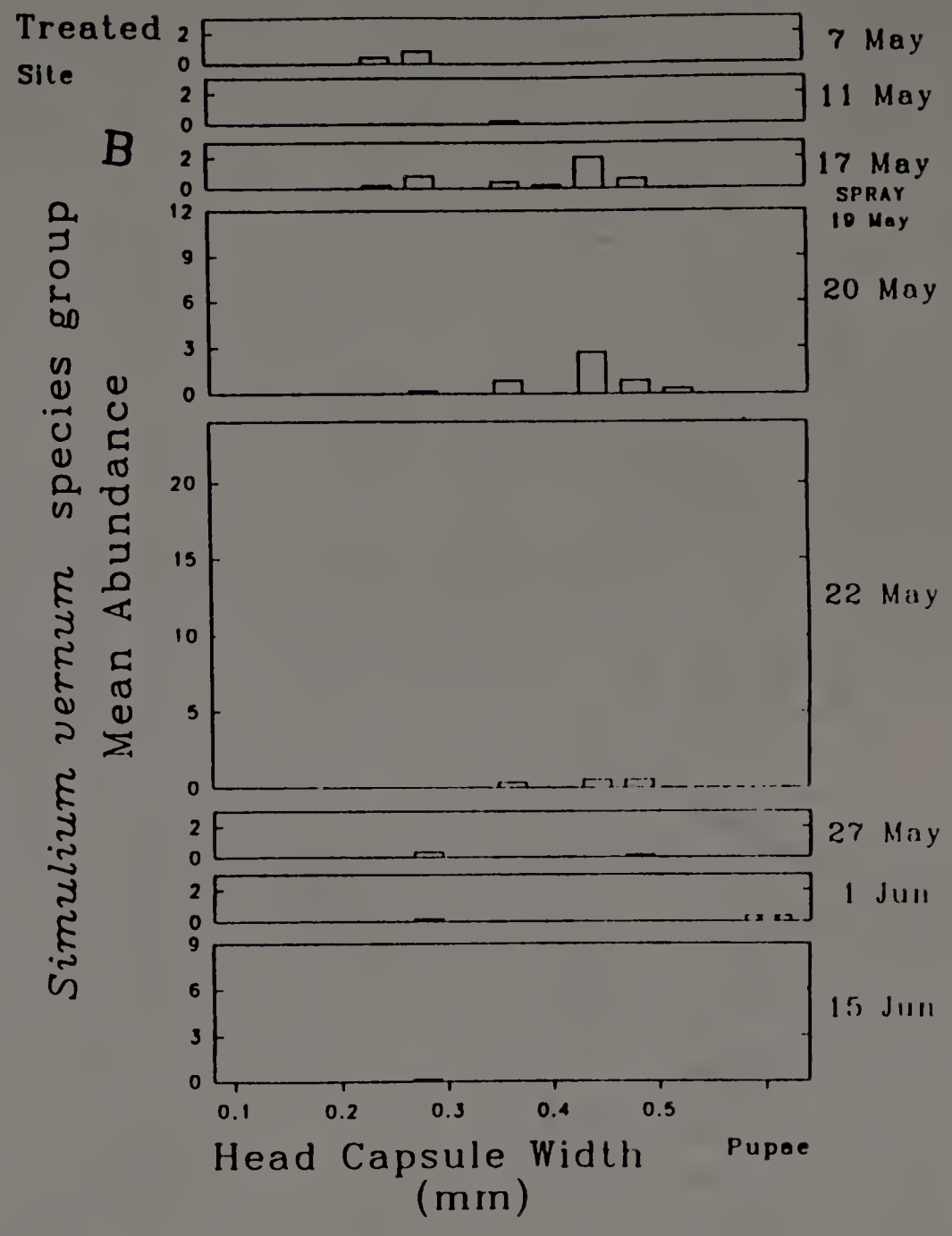
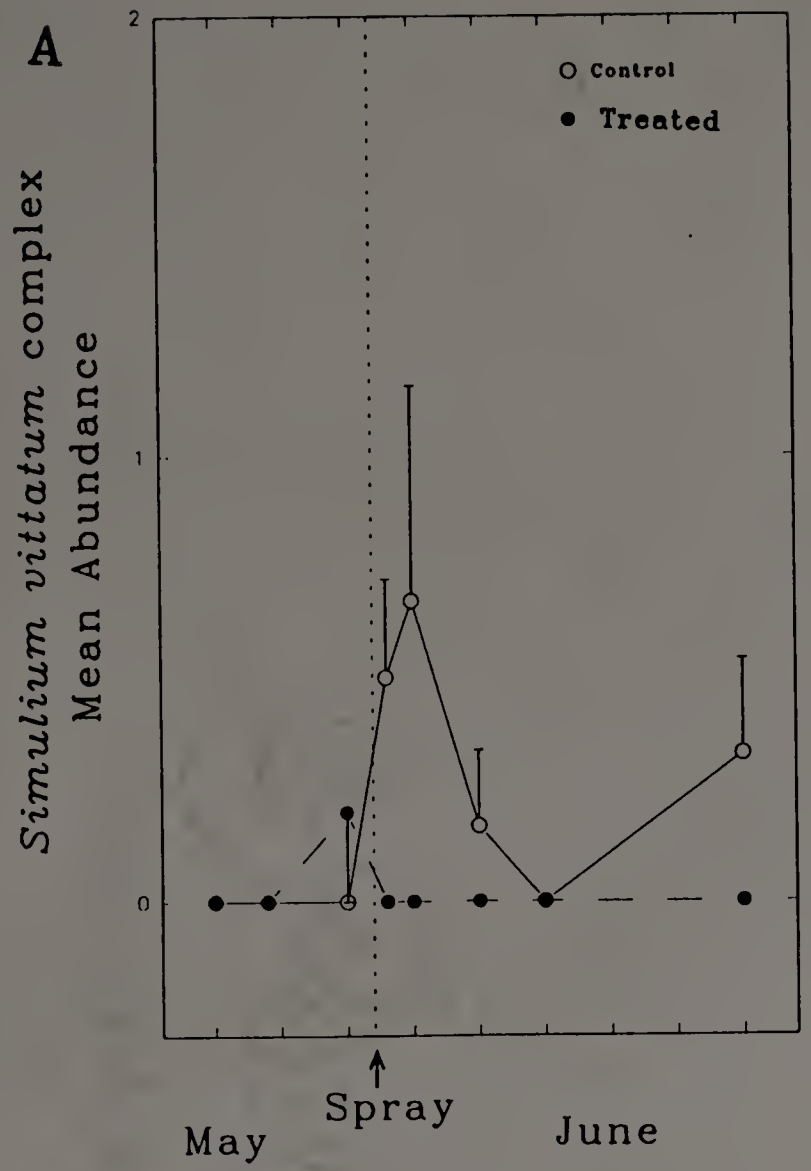

Treated
Site
B
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
Simulium vernum species group
Mean Abundance
Head Capsule Width
(mm)
Pupae

Fig. 33. *Simulium vittatum* complex mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Simulium vittatum complex
Mean Abundance
2
1
0
Control
Treated
Spray
May
June

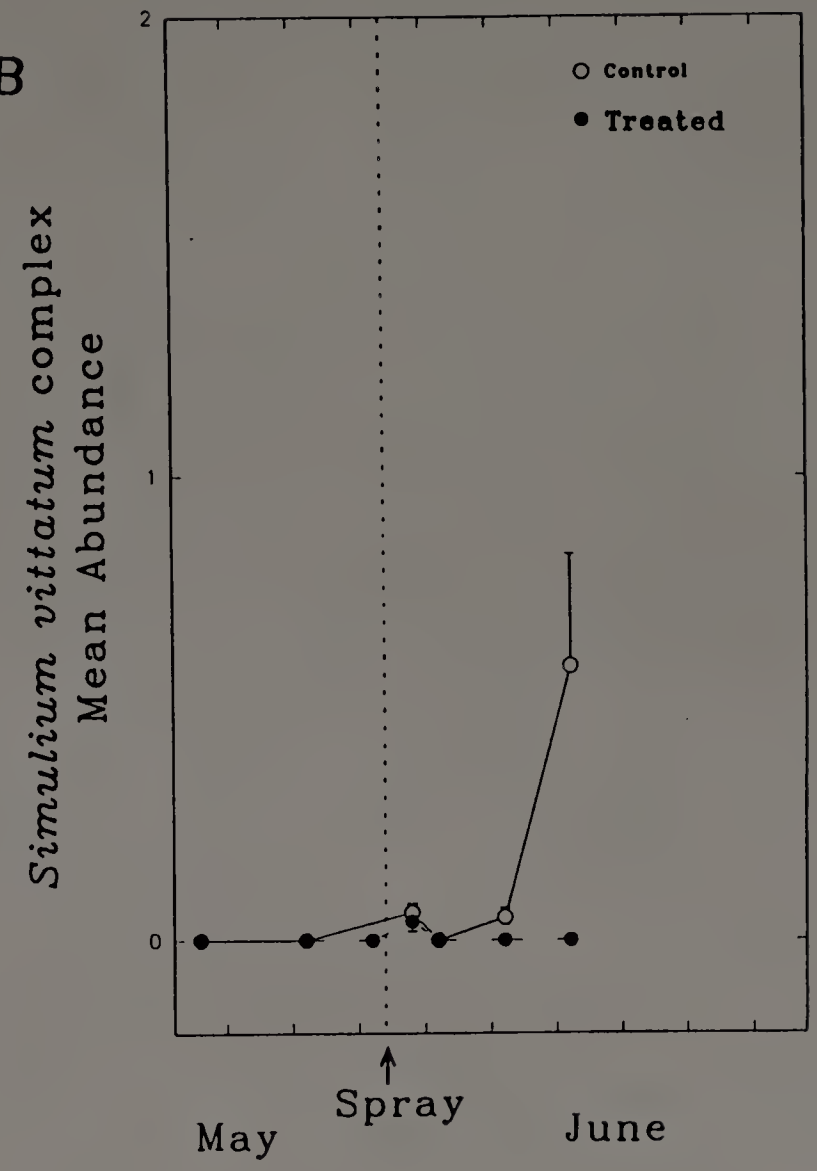
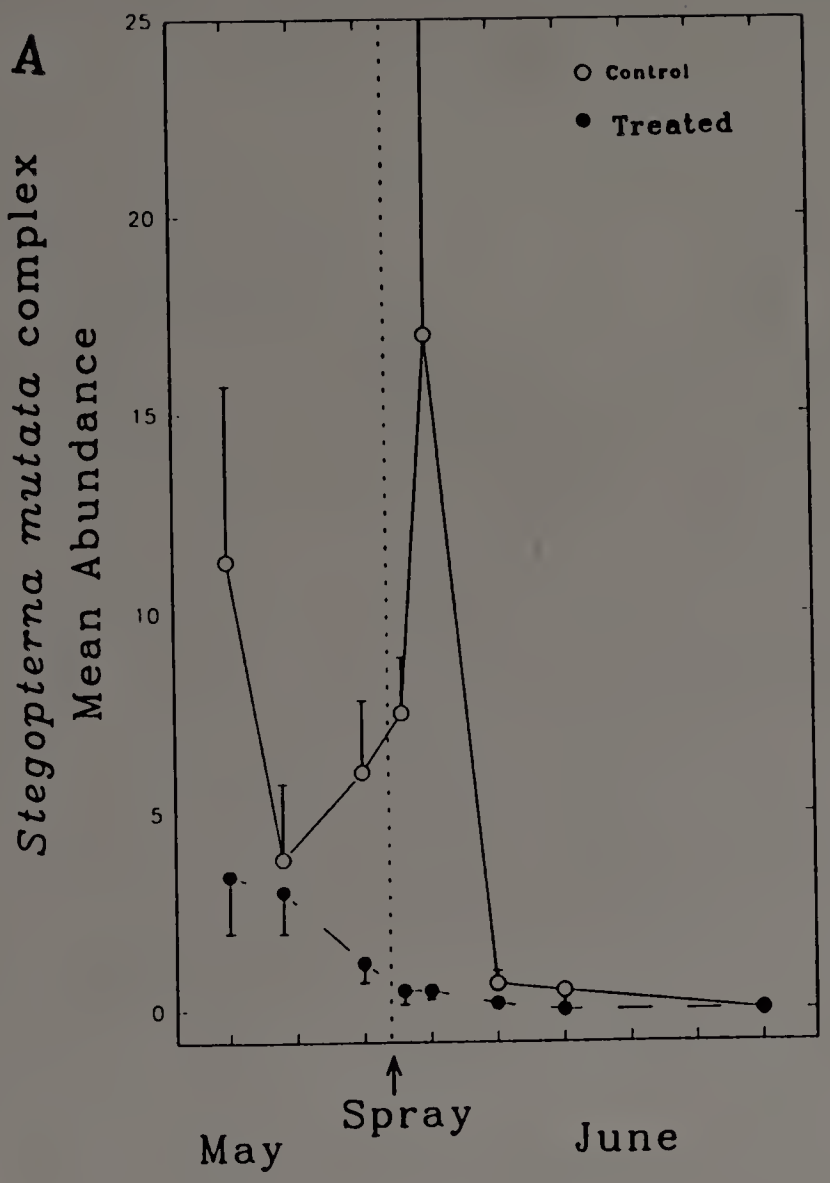

B
Simulium vittatum complex
Mean Abundance
2
1
0
Control
Treated
Spray
May
June

Fig. 34. *Stegopterna mutata* complex mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Stegopterna mutata complex
Mean Abundance
25
20
15
10
5
0
Control
Treated
May
Spray
June

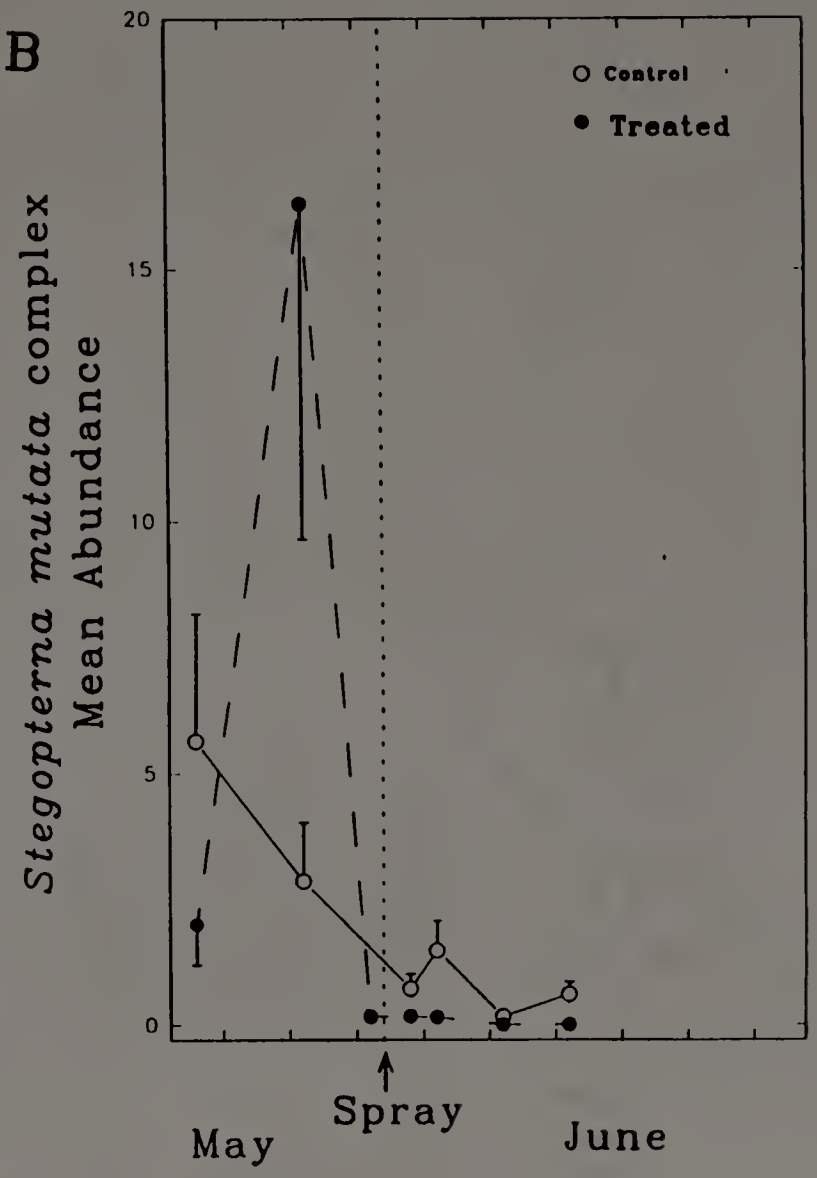

B
Stegopterna mutata complex
Mean Abundance
20
15
10
5
0
Control
Treated
May
Spray
June

Fig. 35. *Stegopterna mutata* complex mean abundance in Surber samples by size class with time, larvae (open bars), pupae (hatched bars), control site (A), treated site (B).

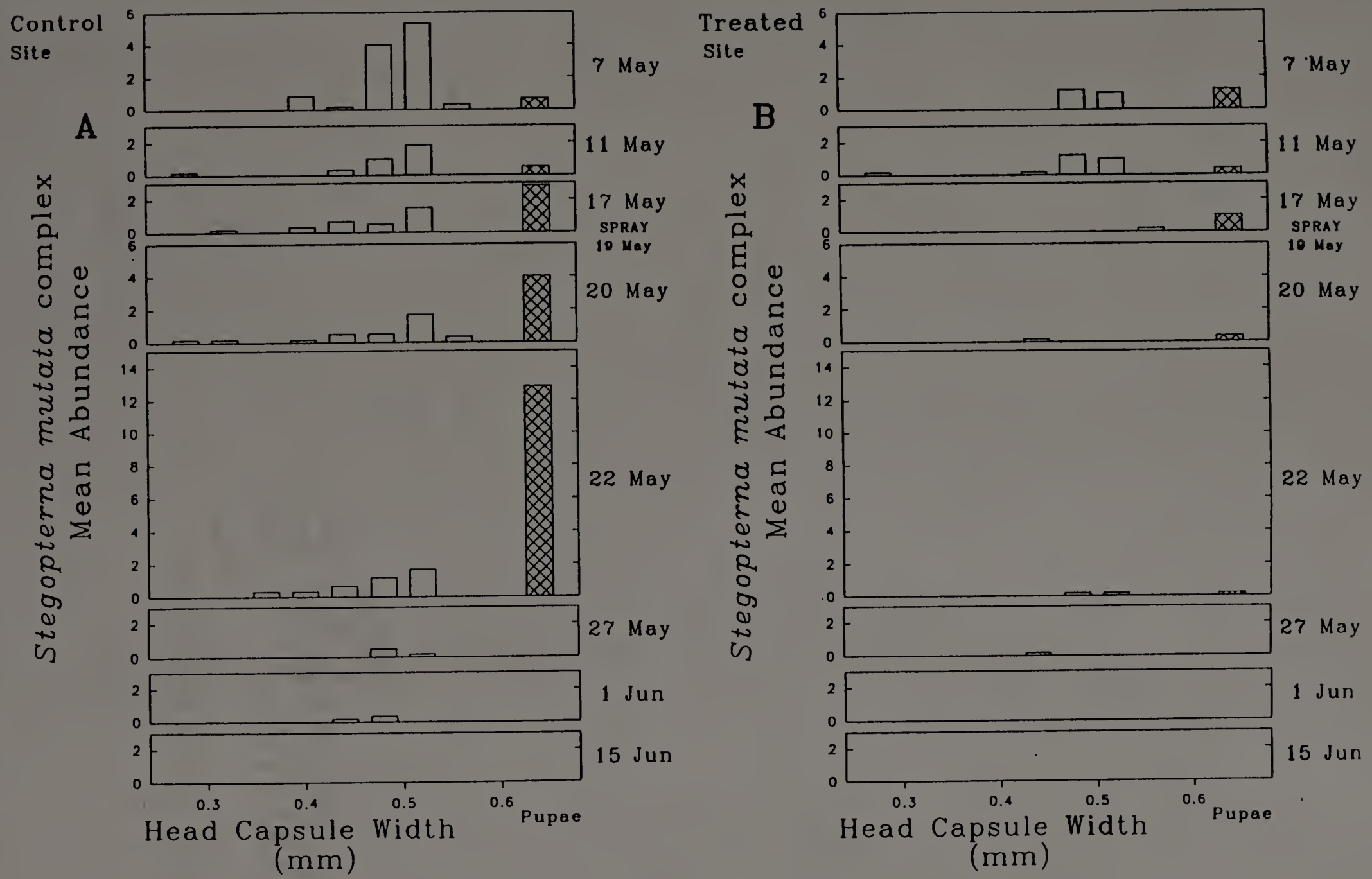

Control
Site
A
Treated
Site
B
Stegopterna mutata complex
Mean Abundance
7 May
11 May
17 May
SPRAY
19 May
20 May
22 May
27 May
1 Jun
15 Jun
0.3
0.4
0.5
0.6
Pupae
Head Capsule Width
(mm)

Fig. 36. Ceratopogonidae mean abundance by site with time, ± 1 S.E., Surber samples.

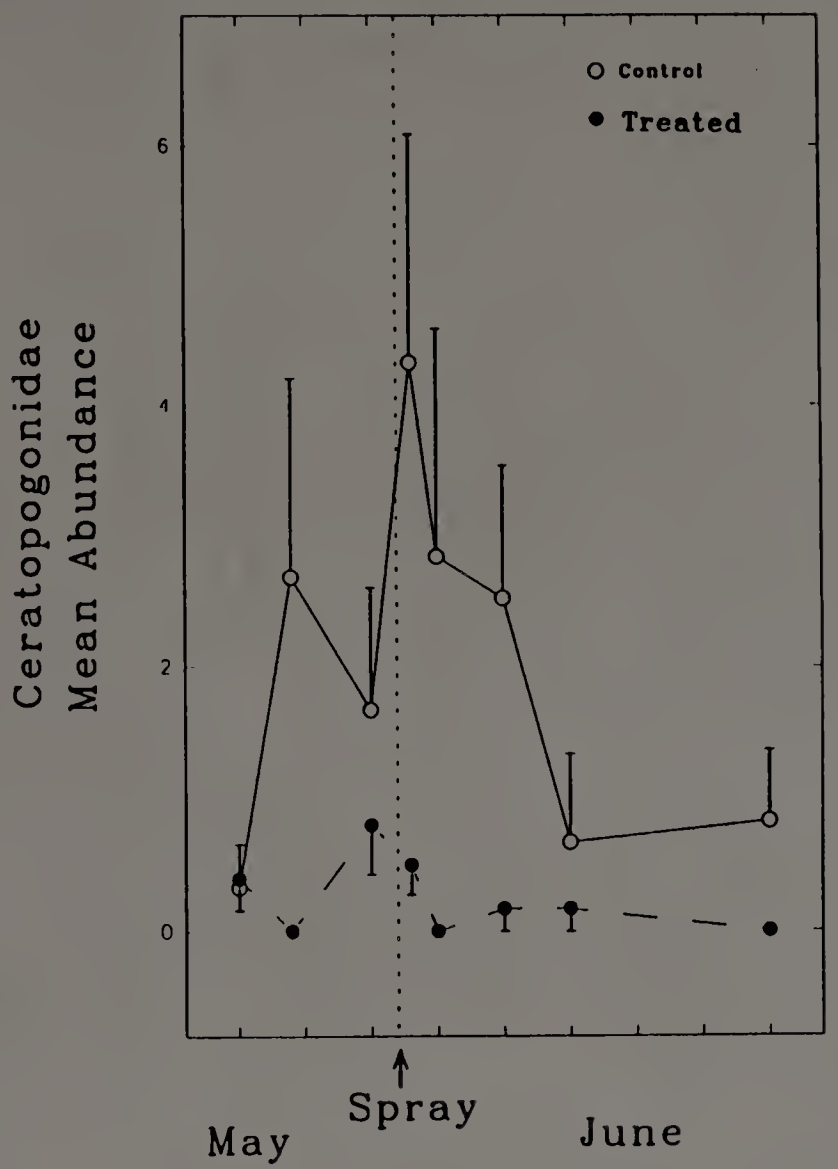
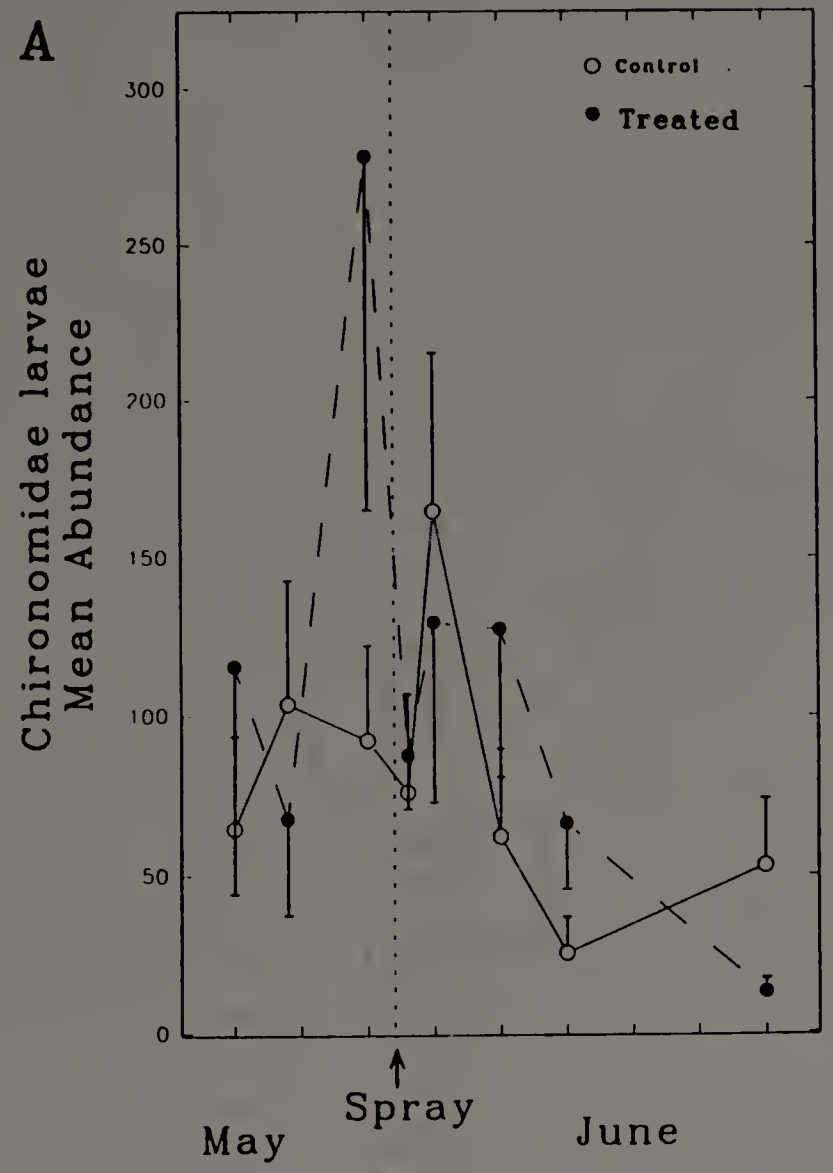

Ceratopogonidae
Mean Abundance
Control
Treated
6
4
2
0
May
Spray
June

Fig. 37. Chironomidae larvae mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Chironomidae larvae
Mean Abundance
Control
Treated
300
250
200
150
100
50
0
May
Spray
June

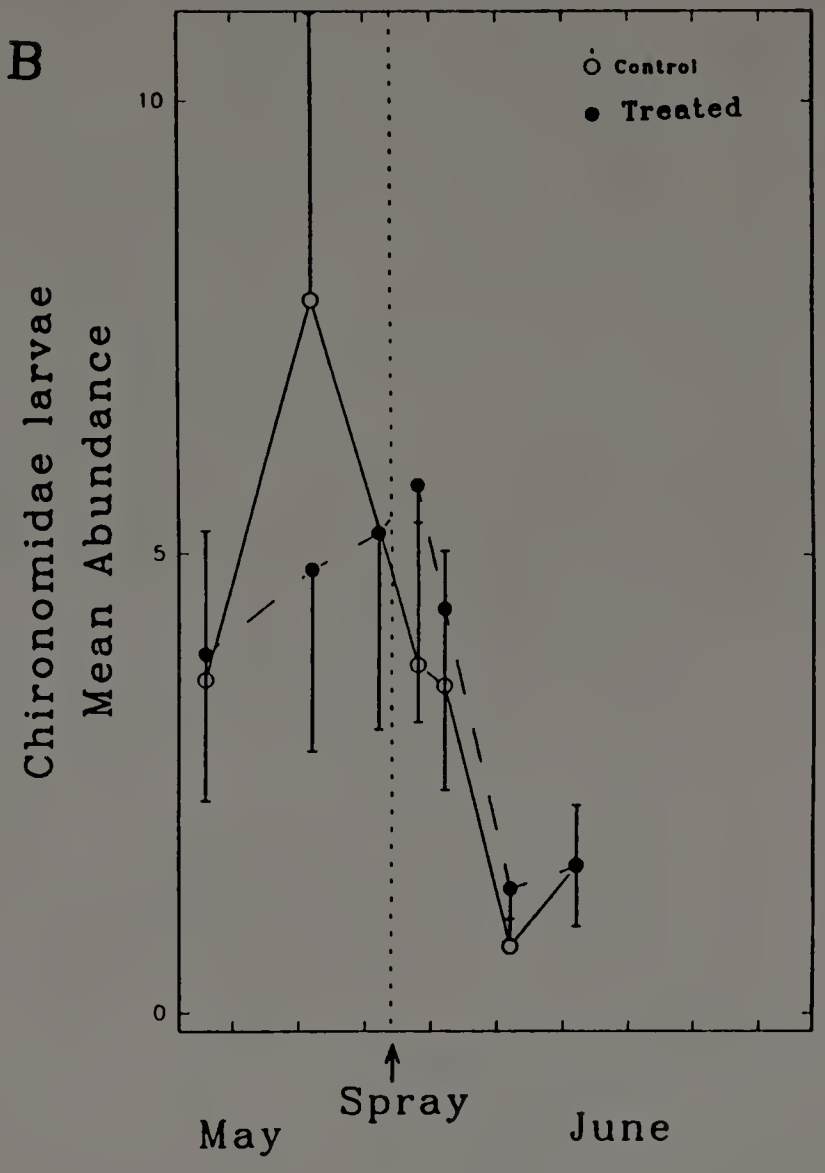
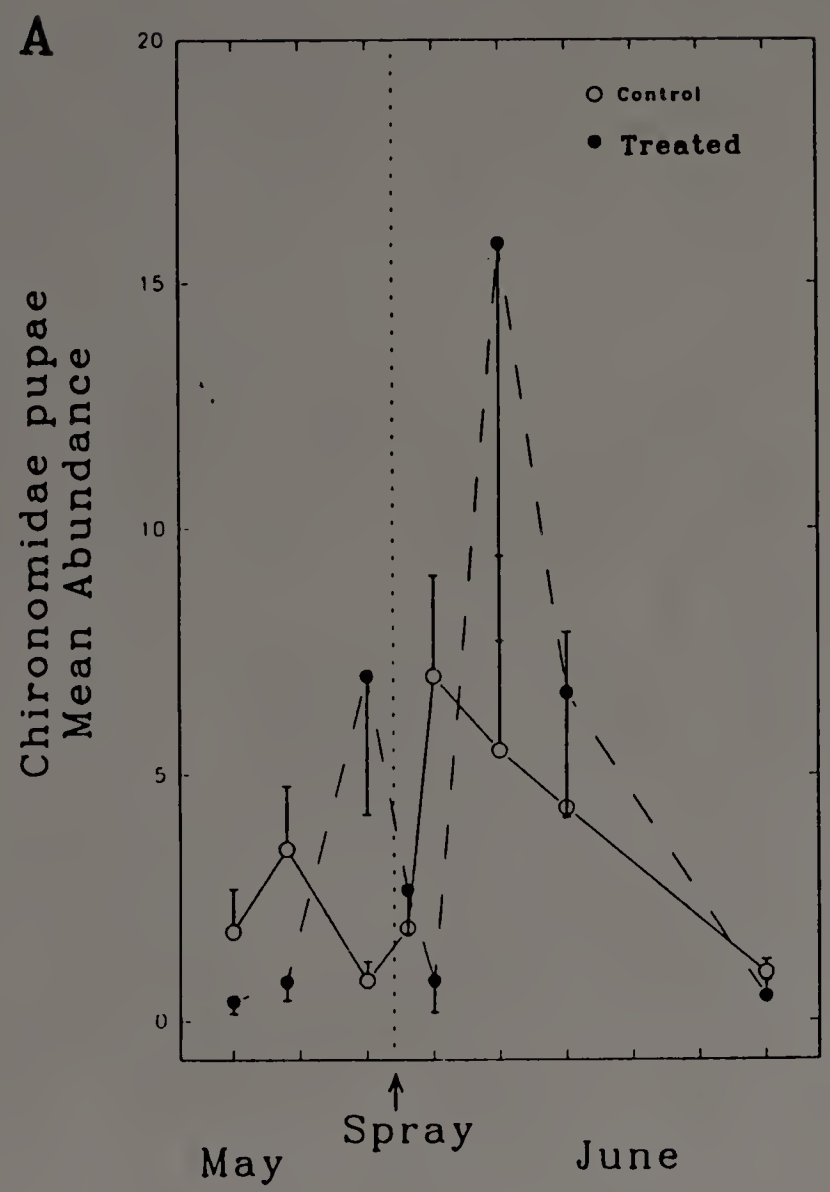
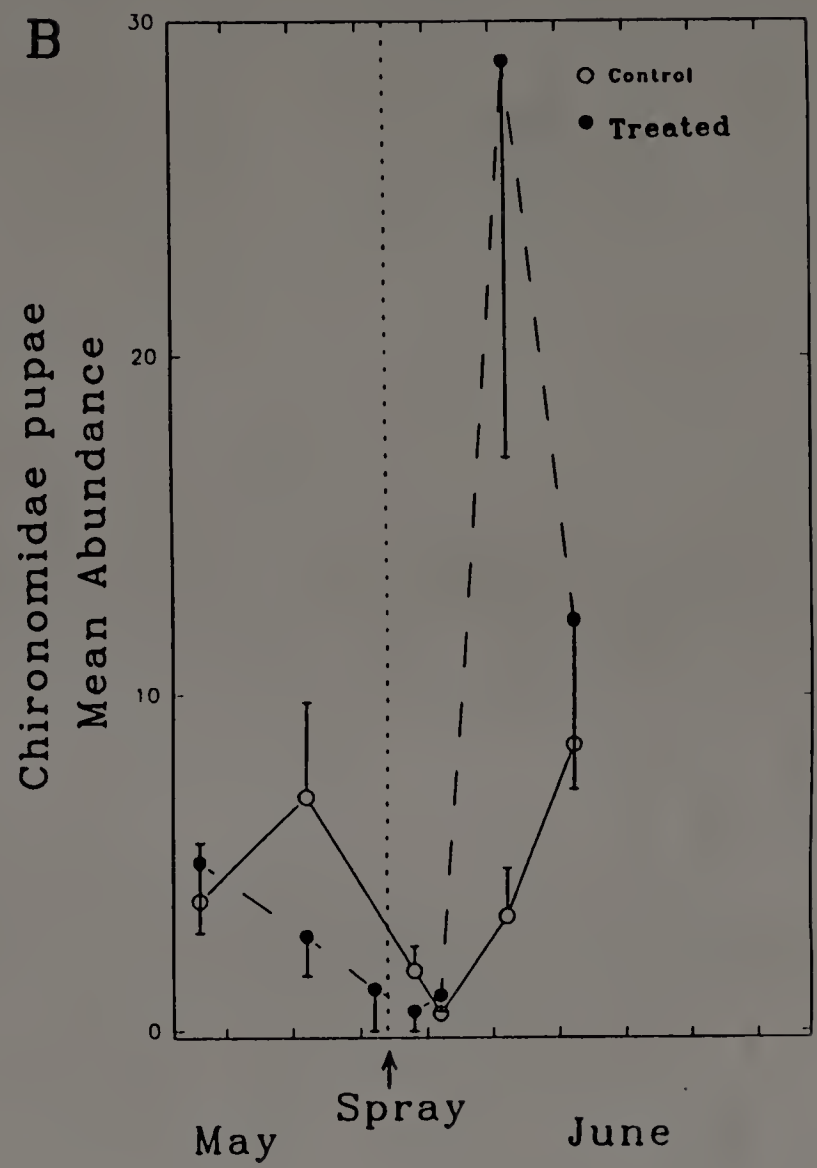

B
Chironomidae larvae
Mean Abundance
Control
Treated
10
5
0
May
Spray
June

Fig. 38. Chironomidae pupae mean abundance by site with time, ± 1 S.E., Surber samples (A), drift samples (B).


A
Chironomidae pupae
Mean Abundance
20
15
10
5
0
Control
Treated
May
Spray
June


B
Chironomidae pupae
Mean Abundance
30
20
10
0
Control
Treated
May
Spray
June

CHAPTER 3

A PRELIMINARY STUDY OF SYNERGISM OF ACID RAIN AND DIFLUBENZURON

Introduction

Diflubenzuron (Dimilin®) was used on over 7 million acres in the U.S. in 1990 to control forest pests, particularly the gypsy moth. This chitin synthesis inhibitor effects insects and other arthropods. It is a restricted use pesticide due to its nontarget effects on aquatic macroinvertebrates (U.S. EPA 1985). The effects of a single aerial application on nontarget aquatic macroinvertebrate communities were reviewed by Eisler (1992). Crustacea and immature insects (especially the true flies, mosquitoes, midges and black flies) are the most sensitive nontarget aquatic organisms to diflubenzuron.

Diflubenzuron is not the only mortality factor aquatic organisms face from human pollution. Acid deposition is a frequent stress factor in freshwater habitats in the Northeast USA. Acidic pulses can drop vernal pools (e.g., temporary, springtime, snowmelt pools) to pH levels below 3.0 (Jackson 1990). Aquatic invertebrates vary in their tolerance to acidification (Zischke et al. 1983). Reduced pH completely eliminates some species (Hall et al. 1980). A combination of stress factors could lead to synergistic effects, over and above the impact seen with a single stressor. The purpose of this study was to determine if there are

synergist effects of diflubenzuron and lowered pH on the mortality of a nontarget aquatic organism.

Materials and Methods

Aedes aegypti (Rockefellar strain) larvae were used as the study organisms. Twenty, 4-day-old larvae were placed in a crystalizing dish with 400 ml reconstituted fresh water (12 mg/L $NaHCO_3$, 7.5 mg/L $CaSO_4 \cdot 2\ H_2O$, 7.5 mg/L MgSO4, 0.5 mg/L KCl; hardness = 10-13 mg $CaCO_3$/L, APHA 1985). The pH was adjusted to 4.5 or 6.6 with an artificial acid rain solution (6.5% and 3% sulfuric and nitric acids, respectively). Mortality levels of control larvae was excessive when pH levels below 4.3 were maintained. Larvae were reared in water at the treatment pH before their experimental use. Larvae at this age are 4-6 days from pupation. Four ml of diflubenzuron in 95% ethanol or, for the control, 95% ethanol alone, was added to each dish. Diflubenzuron concentrations ranged from 0.05 n*M* to 50 µ*M*. Each dose was replicated at least 5 times. The dishes were stacked upon one another and placed in an incubator at 27 ^{o}C. After 96 h, the numbers of live larvae were counted. POLO-PC (Le-Ora Software, Berkeley, CA) was used to analyze the significance of the fit of the data to the probit model, to calculate the LC_{50} values, and to determine if the mortality

curves were significantly different between pH treatments.

Results and Discussion

Increased acidity increased mortality 100 times (Fig. 39). The LC_{50} was 5 n*M* at pH 4.5 and 500 n*M* at pH 6.6. Figure 39 presents the probit mortality curves; dotted lines indicate the 95% confidence interval for each acid treatment. The R^2 values are 0.8235 and 0.8677 for pH 4.5 and pH 6.6, respectively. The mortality curves are significantly different in y intercept (α=0.05) and are parallel.

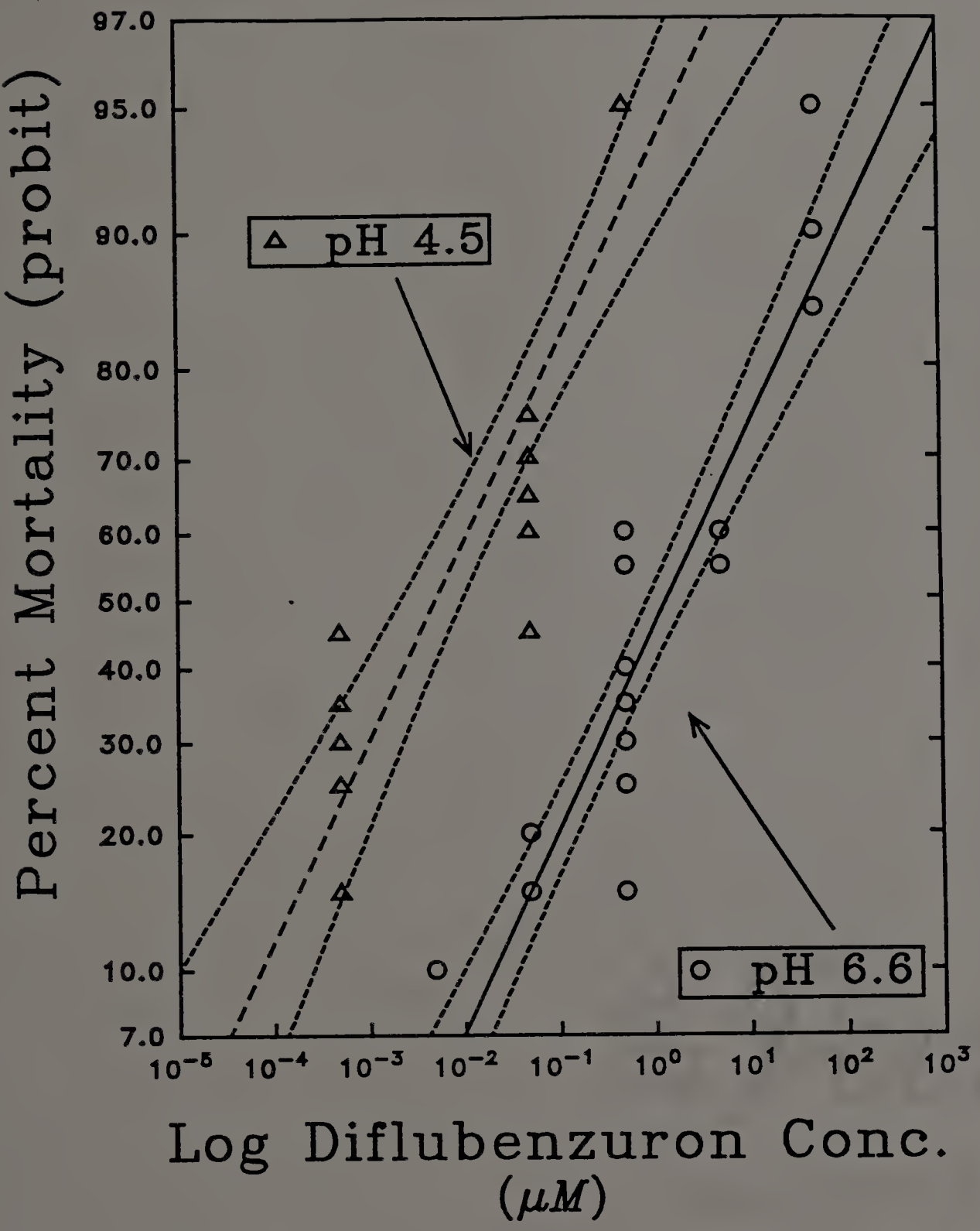
The synergistic action of diflubenzuron with lowered pH has implications for the prediction of the impact of diflubenzuron on nontarget arthropods. Field data obtained in habitats not exposed to acid precipitation may underestimate nontarget mortality in habitats that experience acidic precipitation. The timing of diflubenzuron application in relation to annual acidic pulses could greatly alter nontarget aquatic mortality.

One possible physiological explanation of the effect we have shown is that insect cuticle is more than a simple chitin coat. Cuticle is composed primarily of protein, with $\leq$50% chitin. In immature aquatic insects with nonsclerotized cuticles, most of the protein is noncovalently bound to the chitin

(Andersen 1979). The protein is believed to wrap around chitin microfibrils, providing additional structural support (Kramer et al. 1985). Different cuticular proteins have differing isoelectric points, ranging from pH 3-6 (Andersen 1979) and protein structural integrity is best near its isoelectric point. It is possible that an insect whose cuticle is weakened by the reduced chitin content due to diflubenzuron could still survive since its cuticular proteins continue to provide some support. Diflubenzuron has not been shown to effect cuticular proteins (Grosscurt & Jongsma 1987). However, if protein structure was weakened by a change in pH, then increased mortality would be the expected result.

Another explanation is that ion availability is lowered by lowered pH , and aquatic insects must continuously take-up salts (e.g., Na^{2+}, Ca^{2+}, K^{+}) to survive (Sutcliffe & Hildrew 1986). Ion uptake in *Aedes aegypti* occurs through the anal papillae (Clements 1992). Reduction in chitin content due to diflubenzuron in the papillae may reduce their efficiency. This combined with reduced ion availability could result in mortality not seen at higher pH levels.

Fig. 39. Log dose -- probit response curve to diflubenzuron dose, at pH 4.5 and pH 6.6 for 4 d old *Aedes aegypti* larvae, dashed lines are 95% confidence intervals.


Percent Mortality (probit)
97.0
95.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
7.0
pH 4.5
pH 6.6
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
Log Diflubenzuron Conc.
(μM)

CHAPTER 4

BIOCHEMICAL MODE-OF-ACTION

Introduction

Diflubenzuron (Fig. 40) is a well known chitin synthesis inhibitor (Post & Vincent 1973, Post et al. 1974). However, it has not been shown to inhibit cell-free chitin synthase (Cohen & Casida 1980; Mayer et al. 1980). Its exact biochemical site of action remains unknown (Reynolds 1987). Current theories of diflubenzuron mode-of-action are reviewed in Chapter 1. One mode-of-action hypothesis is that it functions as an inhibitor of a UDP-GlcNAc (Fig. 41) transport mechanism (e.g., an inhibitor of N-acetylglucosaminephosphotransferase (UDP-N-acetylglucosamine:dolichyl-phosphate N-acetylglucosaminephosphotransferase, E.C. 2.7.8.15).

Dol-PP-GlcNAc (Fig. 42) is the carrier of GlcNAc across the membrane of the endoplasmic reticulum for glycoprotein synthesis (Hirschberg & Snider 1987). Dol-P plus UDP-GlcNAc form Dol-PP-GlcNAc and UMP by the action of N-acetylglucosamine-phosphotransferase, a membrane-bound enzyme. This reaction is inhibited by Tunicamycin (Tkacz & Lampen 1985). Tunicamycin also inhibits incorporation of UDP-GlcNAc into chitin in *Triatoma infestans* and *Galleria mellonella* (Quesada Allue 1982). Mayer & Chen (1985) did not find diflubenzuron to inhibit the formation of Dol-PP-

GlcNAc; they found Tunicamycin inhibited the production of this glycolipid.

Chitin synthase, a plasma-membrane bound enzyme, uses UDP-GlcNAc as the precursor for chitin (Hackman 1964). Phosphorylated compounds cannot be transported directly across membranes; facilitated transport is needed. Facilitated transport of chitin precursors across the plasma membrane to the locus of chitin synthase is believed to be necessary (Mitsui et al. 1984, 1985; Grosscurt & Jongsma 1987). Dol-PP-GlcNAc is a possible carrier of GlcNAc to chitin synthase (DeLoach et al. 1981; Grosscurt & Jongsma 1987); it is possible that diflubenzuron impairs this transport. Diflubenzuron is known to inhibit DNA synthesis (DeLoach et al. 1981). Dithiocarbanilates (Fig. 43) also are known to inhibit DNA synthesis, by inhibiting the transport of nucleotides across the cell membrane in leukemia L1210 cells because of a change in membrane permeability (Kessel & McElhinney 1978). Diflubenzuron has been shown to inhibit the transport of nucleotides in melanoma cells (Mayer et al. 1984). Diflubenzuron and dithiocarbanilates are structurally similar (Fig. 40, 43).

The purpose of this study was as follows: 1) determine if dolichol is present in the chitin-synthesizing plasma membrane of *Chironomus tentans* cells, 2) determine if dithiocarbanilates inhibit

chitin synthesis as does diflubenzuron, and 3) determine the physical binding affinity of Dol-PP-GlcNAc for diflubenzuron.

Materials and Methods

Cell Line Methods

Cell Line Culture. *Chironomus tentans* cells (established by Dr. C. Wyss, ETH (Swiss Federal Inst. of Technology) Zürich) were kindly provided by Dr. Steven T. Case (Dept. of Biochemistry, University of Mississippi Medical Cent.). The cells were cultured at 25 ^{o}C in ZW medium (Wyss 1982a) or in IPL-41 medium from Sigma Chemical Corp. (L. Brumley, Dept. of Biochemistry, Univ. of Miss. Med. Cent., pers. commun.). All media were supplemented with 5% (v/v) fetal bovine serum (heat-inactivated), 0.1 mg/L bovine insulin, 60 mg/L penicillin, 100 mg/L streptomycin sulfate, and, for ZW medium, 1 g/L Bactopeptone (Difco). Media osmolarity was adjusted to 340 mOsm; this was the osmolarity of the media sent from Mississippi. Wyss (1982a) suggested an osmolarity of 250 mOsm, however, this did not support cell growth. Cultures were refreshed 1:4 with fresh media once a week (T. Wurtz, Karolinska Institutet, pers. commun.). All chemicals used to prepare the media were of the highest quality available from Sigma Chemical Corp.

Chitin Synthesis Assay: Radiolabelled Precursor.

The chitin synthesis assay with radiolabelled precursor followed that of Spindler-Barth et al. (1989). Cells were concentrated to ca. 100 µg protein/ml either by gravity sedimentation (≥ 4 h) or by centrifugation (1300 *g*, 15 min), and then 1 ml was distributed into sterile microcentrifuge tubes or sterile culture plate depressions. Protein concentration was used as a measure of cell density because this cell line grows in clumps that does not permit cell counting. D-[6-^{3}H]-glucosamine hydrochloride (NEN, 0.5 µCi, 30 Ci/mmol) and unlabelled GlcN were added to a final concentration of 17 µ*M*. Inhibitors were added dissolved in 2.5 µl dimethylsulfoxide. Cells were incubated for 7-8 d (Spindler-Barth et al. 1989).

After incubation, cells were harvested by centrifugation (11,000 *g*, 20 min) and supernatant discarded; then the pellet was washed with 0.1 *M* NaOH, respun (11,000 *g*, 20 min) and supernatant discarded. KOH (500 µl of 1.5 *M*) was added and tubes were heated at 100 °C for 2 h. After cooling, samples were filtered (glass fiber, GF/F) and the filters were washed 10 times with 1 ml of 1.5 *M* KOH, followed by 6 times with 1 ml of 95% ethanol. The filters were dried at 50 °C for 2 h and placed in liquid scintillation counting (LSC) vials. LSC solution, 5 ml, (Universol Cocktail, ICN) was added and vials

were allowed to sit for > 24 h to eliminate chemiluminescence before counting in a liquid scintillation counter (LKB Wallac 1209 Rackbeta).

Chitin Synthesis Assay: Enzymatic Degradation.

The chitin assay was that of Cabib & Sburlate (1988). Cells were harvested by centrifugation (1300 *g*, 10 min). Chitinase (100 µl; from *Serratia marcescens* [Sigma], 1.0 unit/ml in filter-sterilized, 50 m*M* PBS; pH 6.0) was incubated with 100 µl of cells (or chitin suspension as standard) and ß-N-acetylglucosaminase (100 µl; from *Aspergillus niger* [Sigma], 0.1 unit/ml in filter-sterilized, 50 m*M* PBS; pH 6.0) for 12-48 h. Each preparation was analyzed for GlcNAc by the procedure of Reissig et al. (1955) and Reissig & Leloir (1958). Samples had 0.3 ml potassium tetraborate added, and were then heated for exactly 8 min at 100 °C. Samples were cooled, then 3 ml of DMAB reagent (10 g *p*-dimethylaminobenzaldehyde in 100 ml glacial acetic acid with 12.5% (v/v) 10 N HCl), diluted with 9 volumes of glacial acetic acid, was added and mixed. Samples were heated at 37 °C for exactly 20 min and cooled to room temp. Absorbance of each sample was read at 585 nm in a Shimaszu Spectronic 210UV double beam spectrophotometer. Reference blank was unheated sample plus DMAB reagent.

Plasma Membrane Isolation Method. *Chironomus tentans* plasma membranes were isolated with collodial silica according to Chaney & Jacobsen (1983) and Schmidt et al. (1985). Stock suspensions of silica microbeads and anionic polymer were kindly provided by Dr. Bruce Jacobson (Department of Biochemistry, University of Massachusetts, Amherst). All glassware was siliconized (Sigmacote) before use.

Cells in 300-400 ml of culture media were harvested by centrifugation (80 *g*, 10 min), then washed twice with PBS (pH 6.5). Pelleted cells were treated with sterile filtered chitinase (0.1 unit/ml) and ß-N-acetylglucosaminidase (0.1 unit/ml) for 2 h. Cells were again washed (80 *g*, 10 min), viewed by phase microscopy and counted. Four, 10 µl subsamples were taken for protein, glucose-6-phosphatase, cytochrome c oxidase and alkaline phosphatase analyses. Alkaline phosphatase (Lee et al. 1975) was a marker for plasma membrane. Glucose-6-phosphatase (Hodges & Leonard 1974) was a marker for endoplasmic reticulum. Cytochrome c oxidase (Hodges & Leonard 1974) was a marker for mitochondria.

Cells were resuspended in coating buffer (260 m*M* sorbitol, 20 m*M* MES; 300 mOsm, pH 5.5) to a final concentration of 0.2-3.0 x 10^6 cells/ml. After 5 min, cells were centrifuged (80 *g*, 5 min) to remove any broken cells. The pellet was resuspended in the same

volume of coating buffer plus silica suspension. The silica suspension was made by diluting 30% stock silica suspension to 2% with coating buffer, then centrifuging (800 *g*, 5 min) to remove any aggregated material. After 5 min, cells were centrifuged (80 *g*, 5 min) and washed with coating buffer to remove excess silica. Cells were resuspended in coating buffer (pH 5.0) plus anionic polymer (75 µg anionic polymer/ml coating buffer). After 5 min, cells were centrifuged (80 *g*, 5 min) and washed with coating buffer. The supernatant was carefully aspirated and the pellet was washed once with lysis buffer (5 m*M* Tris-HCl, 1 m*M* EGTA, 1 m*M* DTE, 1 µg/ml aprotinin; pH 7.5). Cells were resuspended in fresh lysis buffer to a 50% dilution of concentration after cell quantification. Cells were allowed to sit for 15 min then transferred to a glass homogenizer to rupture any remaining cells and nuclei with 15 strokes. Liberated DNA was digested by addition of 25 m*M* $MgCl_2$ and 50 µg/ml DNAase, incubated at room temperature for 15 min. A few more homogenizer strokes were applied to obtain a homogeneous suspension before centrifugation (400 *g*, 5 min). The pellet was washed 2 times in a larger volume of lysis buffer (approx. a 25% dilution of concentration after cell quantification) to remove DNAase and other contaminants from the plasma membrane sheets.

The sample was further purified by layering over a 70% nycodenz cushion (density ca. 1.45 g/cm^3), 1.5 cm high, then centrifuged (28,000 *g*, 45 min) in a 10.4 ml, 16 x 76 mm Nalgene centrifuge tube. The top layer is be nuclei and whole cell contaminants while the bottom layer is coated plasma membrane sheets. A subsample of the plasma membrane preparation was taken for enzyme assays.

Scanning Electron Microscopy (SEM). Cells or membrane preparations were prepared for SEM by primarily fixing them with a solution of 2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.2) for 1 h. Samples were postfixed in 1% OsO_4 in 0.1 *M* sodium cacodylate buffer for 30 min. Preparations were dehydrated through a series of ethanol solutions for 10 min at each step. Samples were critical point dried, sputter coated and viewed on a JEOL 25S scanning electron microscope at 15 kV.

Protein Determination. Protein was determined with the Bicinchoninic Acid Assay (Smith et al. 1985), using BSA as the standard.

Dolichol Extraction. The plasma membrane samples from all experiments were combined for Dol and Dol-P extraction. Dolichol extraction protocol followed that

of Keller et al. (1989). The combined sample from all plasma membrane experiments (1 ml) was saponified with 1.5 ml 60% KOH and 3.0 ml methanol for 1 h at 100 °C in screw-capped glass tube. Blank samples with internal standards of Dol and Dol-P were used to determine extraction efficiency. Tubes were opened and extracted twice with 4.5 ml diethyl ether stored over 3 Å molecular sieve. Pooled extracts were treated with 5% acetic acid in water and centrifuged (400 *g*, 5 min). The ether layer was dried under N_2 (40 °C) and resuspended in methanol in a siliconized tube (Sigmacote) by vigorous vortexing. Samples were purified on a C18 Sep-Pac column (Waters Associates) equilibrated in methanol. Following application of the sample, the column was first treated with 5 ml methanol to elute squalene, sterols and fatty acids, and then 3 ml hexane/2-propanol/1.4 *M* H_3PO_4 (965:35:0.5, v/v) were added to collect Dol and Dol-P. Columns and siliconized tubes were each washed with 1 ml of hexane to check for retained dolichols. The Dol/Dol-P fraction was concentrated by mixing with an equal volume of water/2-propanol (3:2, v/v), vortexing and centrifuging as needed to break the emulsion. The upper phase and the hexane washes were dried under N_2 (40 °C) and resuspended in HPLC mobile phase.

Binding Assay

Enzyme preparation. The enzyme preparation followed that of Ravoet et al. (1981) and Dallner (1974). Rats, 200-300 g, were given only water overnight before being killed by cervical dislocation. All of the following steps took place on ice. Liver was dissected into small pieces and washed in sucrose buffer (0.25 *M* sucrose, 3 m*M* imidazole-HCl; pH 7.4). Wet weight was determined; then the liver was homogenized with 7-8 strokes in a teflon-glass homogenizer (motor set at 7.5) and brought to a final volume (i.e. the original volume) of 3 ml/g wet weight. The homogenate was centrifuged (9000 *g*, 10 min, 4 ^{o}C) and the supernatant volume was adjusted back to the original volume with sucrose buffer and centrifuged again (105,000 *g*, 60 min, 4 ^{o}C). Pellet was gently resuspended with 1-2 strokes of a glass homogenizer into the original volume and centrifuged (105,000 *g*, 60 min, 4 ^{o}C) to remove proteins adsorbed to microsomes. The pellet was again resuspended into the original volume and subsamples taken for protein assay. This preparation was used for Dol-PP-GlcNAc production. Microsomes not used immediately were stored at -50 ^{o}C covered with a layer of glycerol.

Dol-PP-GlcNAc Production. Methods to prepare Dol-PP-GlcNAc followed that of Ravoet et al. (1981) and

Reuvers et al. (1977). The final volume of the reaction mixture was 50 ml, 200 fold more than that used by Ravoet et al. (1981). Two, 50 ml preparations were run at once and extracted lipids were pooled. A blank using heat-killed microsomes was also run. Amounts listed are final concentrations unless otherwise noted. In each reaction flask, Dol-P in chloroform/methanol (40 µg/ml), 2 m*M* EDTA and 4 m*M* $MnCl_2$ were mixed well in chloroform/methanol (2:1, v/v), then dried under N_2 (40 ^{o}C) and resuspended in aqueous Triton X-100 (0.4%). This solution was mixed well and cooled on ice. The following steps take place on ice unless otherwise noted. Ten ml of enzyme suspension were added and gently stirred for 5 min to permit Triton X-100 to mix well with the enzyme. Thirty ml of reaction buffer (80 m*M* MES-glygly-KOH (pH 7.5), 28 µ*M* UDP-GlcNAc, 10 m*M* $MgCl_2$, 2.5 m*M* dithiothreitol and 2 m*M* ATP) were added. The reaction mixture was incubated in water bath at 17 ^{o}C for 15 min. Reaction was stopped by addition of 375 ml (i.e. 7.5 volumes of 50 ml solution) methanol followed by 750 ml (i.e. 15 volumes of 50 ml solution) chloroform.

Lipid isolation & preparation of alkali-stable lipid extract. The lipid extraction procedure followed that of Reuvers et al. (1977). The reaction mixture containing 7.5 volumes of methanol and 15 volumes of

chloroform was shaken at 30 °C for 1 h, then centrifuged (1000 *g*, 20 min). The supernatant was washed with 0.2 volumes 0.9% NaCl (Heifetz & Elbein 1977) in a separatory funnel. After phase separation, the phase boundary layer was washed 10 times with 2 ml of Folch's theoretical upper phase (chloroform/methanol/water, 3:48:47, v/v). The lower phase was collected and dried under reduced pressure (Rotovap) at 30 °C. The lipid extract was saponified by dissolving in 0.2 *M* NaOH in 50% 1-propanol and heating (60 °C, 20 min) in a sealed tube. The sample was neutralized with 5 volumes of 0.04 M HCl followed by 20 volumes of chloroform/methanol (2:1, v/v). After phase separation, the lower phase was washed 10 times with 2 ml of Folch's theoretical upper phase. The lower phase was then dried under reduced pressure (Rotovap) at 30 °C and dissolved in chloroform/methanol (2:1, v/v). This preparation is the alkali-stable lipid extract.

Further purification of the alkali-stable lipid extract. The alkali-stable lipid extract (1-2 ml) were placed on a ion exchanger column (DEAE cellulose column, 10 cm times 1 cm, in acetate form, Christie 1982) in chloroform/methanol (2:1, v/v). Column was washed with 40 ml of chloroform/methanol (2:1, v/v), 40 ml of methanol, 40 ml of 10 m*M* ammonium acetate in

chloroform/methanol (2:1, v/v) and 50 ml of 30 m*M* ammonium acetate in chloroform/methanol (2:1, v/v). The dolichol pyrophosphate-linked sugars elute in the 30 m*M* salt fraction while monophosphate-linked polyprenoid glycolipids elute in the 10 m*M* salt fraction (Reuvers et al. 1977; Keller et al. 1985). The first fraction, 40 ml of chloroform/methanol (2:1, v/v), was dried under reduced pressure (Rotovap) and resuspended in approx. 2 ml chloroform/methanol (2:1, v/v) and again sent through DEAE cellulose (acetate form) column. The column was washed as above and the 30 m*M* salt fraction was combined with the first 30 m*M* salt fraction. This was done in case the column was overloaded. The 30 m*M* ammonium acetate fraction was washed with 0.2 volumes of water to desalt and then dried under reduced pressure (Rotovap) at 30 ^{o}C. The extracts were dissolved in chloroform/methanol (2:1, v/v) and further purified by TLC.

Thin layer chromatography (TLC). TLC of lipid extract was conducted in the following solvents: Solvent A, chloroform/methanol/water (60:35:6, v/v); Solvent B, chloroform/methanol/acetic acid/water (50:25:7:3, v/v); Solvent C, chloroform/methanol/ammonium hydroxide (75:25:4, v/v). Whatman Silica K6F plates (with or without fluorochrome) were used. Spots were visualized under

UV light or with Rhodamine 6G (0.01% w/v, H2O) spray reagent under UV light (Christie 1982).

To recover spotted compounds, the plates were scraped, scrapings placed in a glass column and washed with 50 ml of chloroform/methanol (2:1, v/v). The eluate was dried under reduced pressure (Rotovap, 30 ^{o}C) and, if fluorochrome plates or Rhodamine 6G spray were used, then respotted on TLC plate without visualization. Plates were run in Solvent A; contaminating visualization reagents travelled with the solvent front. Compound was again scraped off at the appropriate R_f position. Scrapings were again eluted with chloroform/methanol (2:1, v/v), dried under reduced pressure and resuspended in chloroform/methanol (2:1) or HPLC mobile phase (for quantification).

Indirect Immunoassay of Diflubenzuron (ELISA).

Indirect immunoassay of diflubenzuron was undertaken as an alternative to HPLC quantification. This procedure is depicted in Fig. 44. Coating antigen and primary antibody serum were gifts kindly provided by Dr. Bruce D. Hammock (Department of Entomology and Environmental Toxicology, University of California, Davis). Coating antigen (N-(Carboxypropyl)diflubenzuron--ovalbumin) was diluted 1:500 in coating buffer (0.1 *M* sodium carbonate/bicarbonate buffer; pH 9.8) and 100 µl was added to each well in a microtitre plate made of

polystrene. The plate was incubated for 10 h at 37 °C then washed three times with PBS-Tween (8.0 g/L NaCl, 0.2 g/L KH_2PO_4, 2.8 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g/L KCl, 0.5 ml/L Tween 20, 0.2 g/L sodium azide; pH 7.4). The blocking agent (200 µl, 2.5% gelatin solution) was added, incubated for 30 min, followed by 3 washes of PBS-Tween. Primary antibody was diluted 1:500 with PBS-Tween containing 1% BSA. Diluted primary antibody (100 µl) was added to each well and incubated 2 h at room temp. Plates were washed 3 times with PBS-Tween. Secondary antibody (100 µl, IgG alkaline phosphatase, 1:3000 dilution) was added to each well and incubated for 2 h at room temp. The plate was washed 3 times with PBS-Tween. The substrate solution (100 µl, *p*-nitrophenyl phosphate, 1 mg/ml, in 10% (w/v) diethanolamine buffer; pH 9.8) was added to each well. The reaction was stopped after 30 min with 25 µl 3 N NaOH. The plates were read at 405 nm.

To quantify diflubenzuron, the primary antibody (1 ml) was incubated overnight with varying amounts of diflubenzuron (or inactive analogues, Fig. 45, kindly supplied by Dr. K. Nishimura, Department of Agricultural Chemistry, Kyoto University) in 20 µl acetonitrile. Increasing concentrations of diflubenzuron inhibited color production, by reducing the amount of primary antibody binding to the coating antigen.

Binding Assay Design. To determine the binding affinity of diflubenzuron for Dol-PP-GlcNAc, the following experiment was planned. C_{18} extraction disks (Empore solid support disks) held on a siliconized (Sigmacote) 25 mm glass filter holders were prewashed with 10 ml of methanol followed by 10 ml of acetonitrile/water (75:25, v/v). A known quantity of Dol-PP-GlcNAc (with Dol-PP-$(GlcNAc)_2$) should be applied to the disk followed by molar equivalent of diflubenzuron. C_{18} disk then washed with 15-20 ml of acetonitrile/water (75:25, v/v) and fractions collected. Diflubenzuron should be quantified in each fraction. The experiment would be repeated with Dol or Dol-P on the disk rather than Dol-PP-GlcNAc, and with inactive analogues of diflubenzuron. Comparisons of the slope of the regression line of the concentration of diflubenzuron versus fraction number would be undertaken to denote binding affinity differences.

High pressure liquid chromatography (HPLC)

HPLC quantification of dolichol was after Keller et al. (1989). The mobile phase was methanol/2-propanol/hexane/85% phosphoric acid (400:400:200:1, v/v), in a C_{18} Zorbax column (DuPont Chromatography, #880952.702) equilibrated for > 3 h. A Shimadzu SPD-2A UV detector was set at 210 nm with

fullscale at 0.04 absorbance units and a flow rate of 1.1 ml/min was maintained with a Waters pump (model 6000A). Multiple dolichol homologues were separated by this reverse phase HPLC method (Keller et al. 1985).

HPLC quantification of diflubenzuron followed U.S. EPA (1982). The mobile phase was acetonitrile/water (60:40, v/v), in a C_{18} Zorbax column (DuPont Chromatography, #880952.702), precleaned with methanol, methylene chloride, hexane, methylene chloride, methanol, acetonitrile solvent series. A Shimadzu SPD-2A UV detector was used, set at 254 nm, fullscale at 0.01 absorbance units and a flow rate of 1.1 ml/min on a Waters pump (model 6000A).

Results

Cell Line

Chironomus tentans cells grew at a low rate in both ZW and IPL-41 media. Cell protein concentration ranged from 1-3 µg protein/ml of media in cultures at maximum apparent growth (determined by visual observation of culture turbidity). Radiolabel incorporation into chitin was low and no inhibition by diflubenzuron was seen in both microcentrifuge tubes or culture plates (Table 5). Incorporation rates were 1 to 2 orders of magnitude greater in the Londershausen et al. (1988) study of chitin synthesis in *C. tentans* cell culture (Table 5). The presence of chitin in cell

culture was demonstrated by enzymatic degradation of cells with chitinase plus ß-acetylglucosaminase. GlcNAc content increased with time. No comparison of dithiocarbanilate inhibition to diflubenzuron inhibition was conducted due to low levels of radiolabel incorporation (Table 5).

In plasma membrane isolation procedures, cell concentrations ranged from 3 x 10^5 to 2 x 10^6 cells/ml, with a final volume of ca. 1 ml. Protein content was ca. 60 µg/ml and 10 µg/ml, for whole cells and plasma membrane preparation, respectively. Alkaline phosphatase assay was conducted on whole cells and on plasma membrane preparation. No activity was seen with plasma membrane preparations. Probable cause was that all isolation steps occurred at room temperature (> 3 h). No other enzyme assay was attempted. SEM photographs of cells before and after chitinase treatment and plasma membrane preparations are depicted in Fig. 46. Whole cells were present in an extracellular matrix that largely disappeared after treatment with chitinase (Fig. 46 A & B). Sheets of putative plasma membrane, without presense of whole cells, were seen following the silicia coating, plasma membrane purification procedure (Fig. 46 C).

The dolichol standard curve at the time of *C. tentans* dolichol quantification is shown in Fig. 47. The standard curve varied over the life time of the

column. Therefore, standard quantification was repeated as necessary. No dolichols were found in extracted plasma membrane from *C. tentans*. Whole *Chironomus tentans* cells contained ca. 100 ng Dol/200 µg protein (summed peak area = 2250590; Fig. 48).

Binding Assay

The results of thin layer chromatography of final lipid extract are shown in Table 6. Large quantities (ca. 1 ml) of extract had to be applied to the plate to cause a visible spot. Identification of spots was based solely on relative R_f positions in different solvent systems compared with reported R_f positions.

Dol, Dol-P, Dol-PP-GlcNAc and Dol-PP-$(GlcNAc)_2$ homologues coeluted from the HPLC columns with retention times of ca. 8.2, 9.3, 10.5, 11.8, 13.5, 15.3 min (Fig. 49). Retention times varied, especially as column use increased. Identification of peaks is based solely on elution position of standards (or previous identification on TLC). Blank preparations (run with heat-killed microsomes plus Dol-P) did not contain any Dol-P at the end of extraction procedure. This indicated that HPLC peaks found in lipid extracts after live enzyme incubation were due to Dol-PP-GlcNAc and/or Dol-PP-$(GlcNAc)_2$ rather than Dol-P passing through the extraction procedure. One hundred ml

reaction mixture resulted in ca. 1 nmol Dol-PP-GlcNAc production (mixed with Dol-PP-$(GlcNAc)_2$).

HPLC response to diflubenzuron was linear over the range of 100 - 300 ng. Below 25 ng, the 95% confidence interval was ca. ± 40% (Fig. 50). ELISA quantification of diflubenzuron was possible to 0.5 ng/well (Fig. 51). This would be equivalent to a minimum of 187.5 ng diflubenzuron/ml because the primary antibody solution can only withstand 20 µl of acetonitrile (the solvent for diflubenzuron) per ml. Concentrations of diflubenzuron above 5 ng/well produced the maximum inhibition of color production seen in this study (Fig. 51). Inactive diflubenzuron analogues (#16 and #18 from Nakagawa et al. 1992, Fig. 45; 40 ng/well) cross-reacted to give a positive response in ELISA assay (Fig. 51). Therefore, this assay could not distinguish inactive analogues from diflubenzuron, and therefore this assay could not be used when diflubenzuron was mixed with one of these inactive analogues. These inactive analogues are not normally present as a contaminant; they are used as experimental compounds in binding affinity studies.

It was determined that Dol and Dol-P remains on the C18 disk with acetonitrile/water (75:25, v/v) as solvent while > 98% of diflubenzuron elutes in 15 ml (Fig. 52).

Discussion

The low growth rate of *Chironomus tentans* is a likely explanation for the low GlcN incorporation rates and the lack of diflubenzuron inhibition (Table 5). Londershausen et al. (1988) reported inhibition rates of 70% with 1 µ*M* SIR 8514 (another benzoylphenylurea) and incorporation rates of 4000 cpm/100 µg protein/6 day incubation using this cell type and the same assay system, in microcentrifuge tubes. This is an incorporation rate 20 times higher than found in the present study. Cell morphology differed between that reported in the Spindler-Barth/Londershausen laboratory (Spindler-Barth et al. 1989) and the culture received from Mississippi. The Spindler-Barth culture grew in vesicles (i.e. round clumps of cells) with chitinous-layers surrounding clumps of cells in each vesicle. The Mississippi culture grew in small clumps, with strands of suspected chitinous compounds coming out of the clumps. These strands disappeared after treatment with chitinase. The rate of chitin synthesis and the cell growth rate may have been different in these two strains of *C. tentans*.

Chaney & Jacobson (1983) suggested a concentration of 3 x 10^6 cells/ml of coating buffer before silica suspension was added for plasma membrane isolation; in these experiments the cell concentration was up to 10 times less than the suggested level. This may have

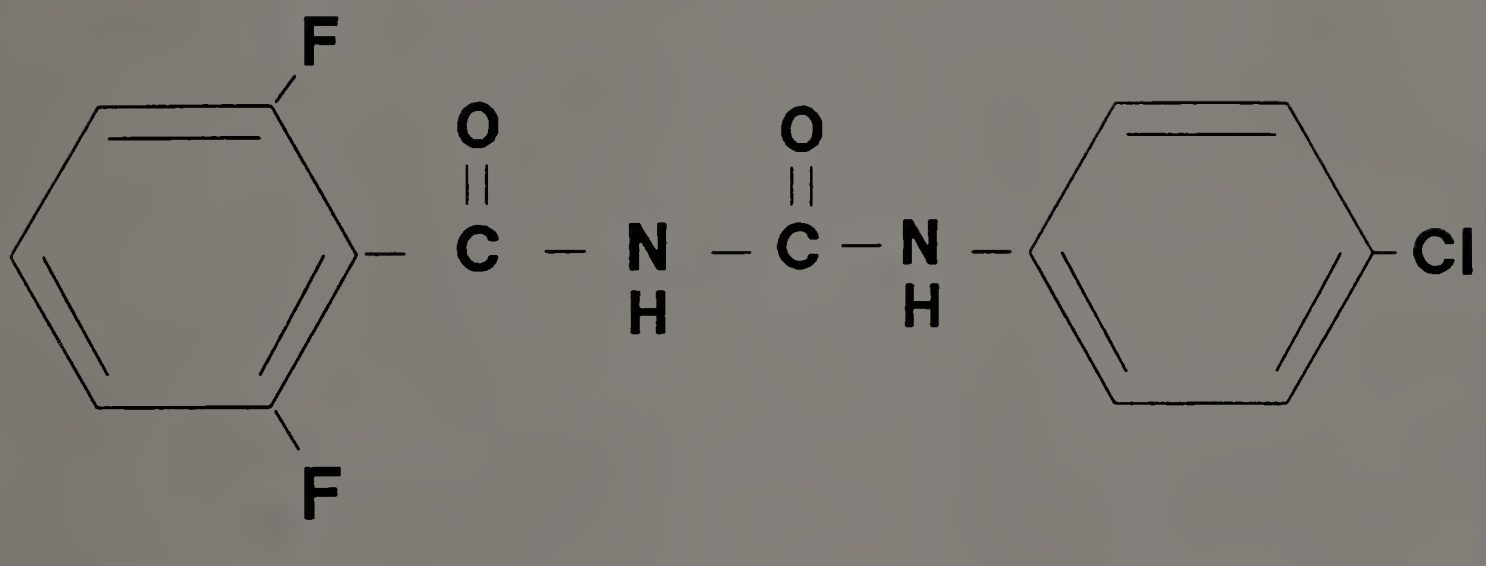
effected the results. The low amount of material in the plasma membrane preparations limited my ability to identify any dolichols that may have been present. Additionally, it is not possible to be certain that the plasma membrane preparation was indeed plasma membrane. The presence of sheets of membrane is suggested in the SEM photographs (Fig. 46C), however, the "pebbly texture" reported in silica-polymer coated membranes by Stolz & Jacobson (1992), Chaney & Jacobson (1983) and Schmidt et al. (1985) did not appear.

Successful completion of the binding affinity study of diflubenzuron with Dol-PP-GlcNAc requires using molar equivalents of diflubenzuron with Dol-PP-GlcNAc (to avoid too high a background of diflubenzuron). Dol-PP-GlcNAc cannot be produced in large amounts (e.g., 1 nmol/7 d lab work). If low amounts of Dol-PP-GlcNAc are used to determine binding affinity with diflubenzuron, then low amounts of diflubenzuron must be able to be quantified. Therefore, the assay for diflubenzuron must be quite sensitive. Neither HPLC nor ELISA produces a sensitive enough analysis for the binding study. Gas chromatography would require a multi-step derivitization procedure but would provide 10 times greater sensitivity than HPLC (U.S. EPA 1985). The necessary derivitization makes this impractical. The only reasonable method would be using radiolabelled

diflubenzuron, which was not available from the manufacturer, nor from any federal laboratories.

Fig. 40. Chemical structure of diflubenzuron, a chitin synthesis inhibitor in arthropods.

Dimilin®(=diflubenzuron)



N-[{(4-chlorophenyl)amino}carbonyl]-2,6-difluorobenzamide

Fig. 41. Chemical structure of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a substrate for chitin systhesis.

UDP-N-Acetyl-D-Glucosamine

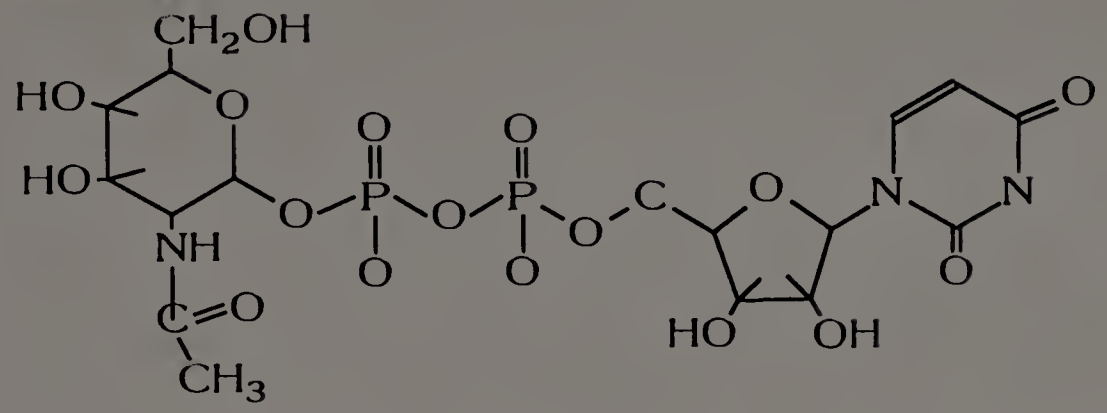
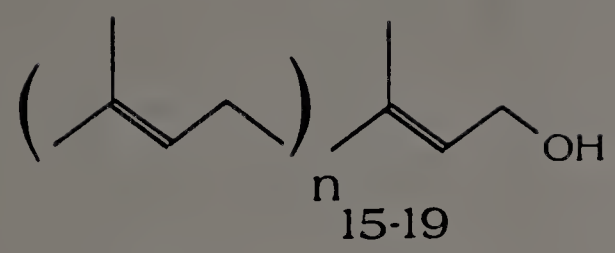
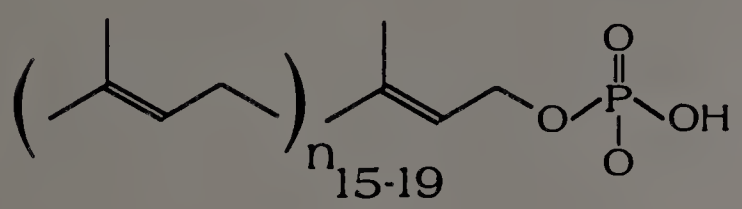


Fig. 42. Chemical structure of dolichol pyrophosphate N-acetylglucosamine (Dol-PP-GlcNAc), a GlcNAc transport substrate.

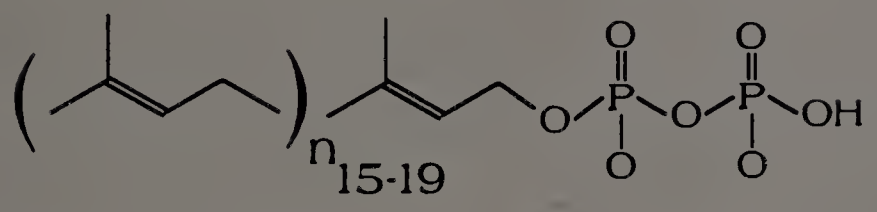
Dolichol



Dolichol Monophosphate



Dolichol Pyrophosphate



Dolichol Pyrophosphate N-Acetyl Glucosamine

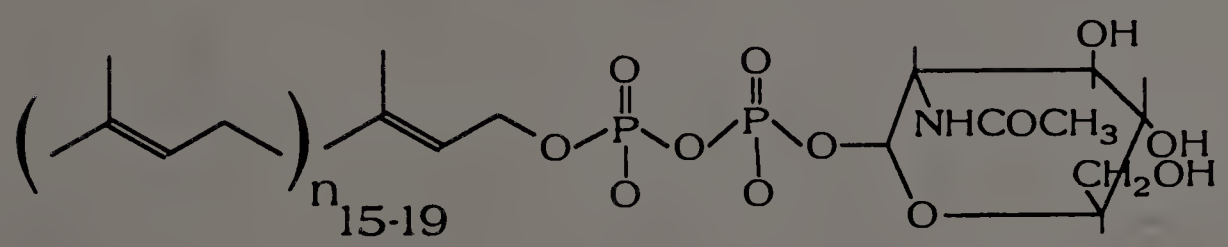
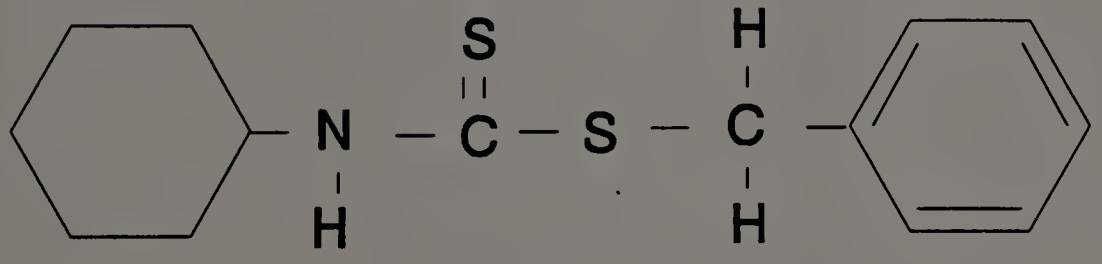


Fig. 43. Chemical structure of two dithiocarbanilates known to inhibit nucleoside transport in leukemia L1210 cells by changing surface hydrophobicity of the membrane (Kessel & McElhinney 1978).

Dithiocarbanilates

Kessel & McElhinney 1978



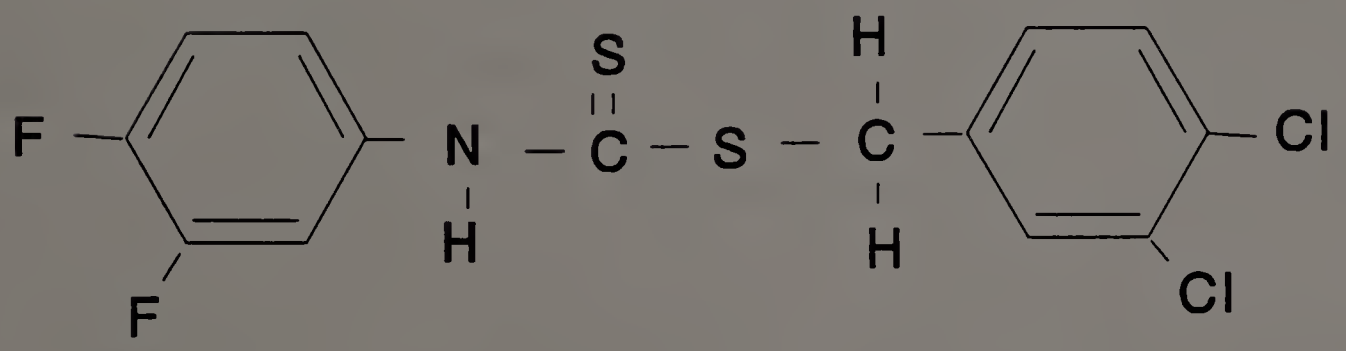
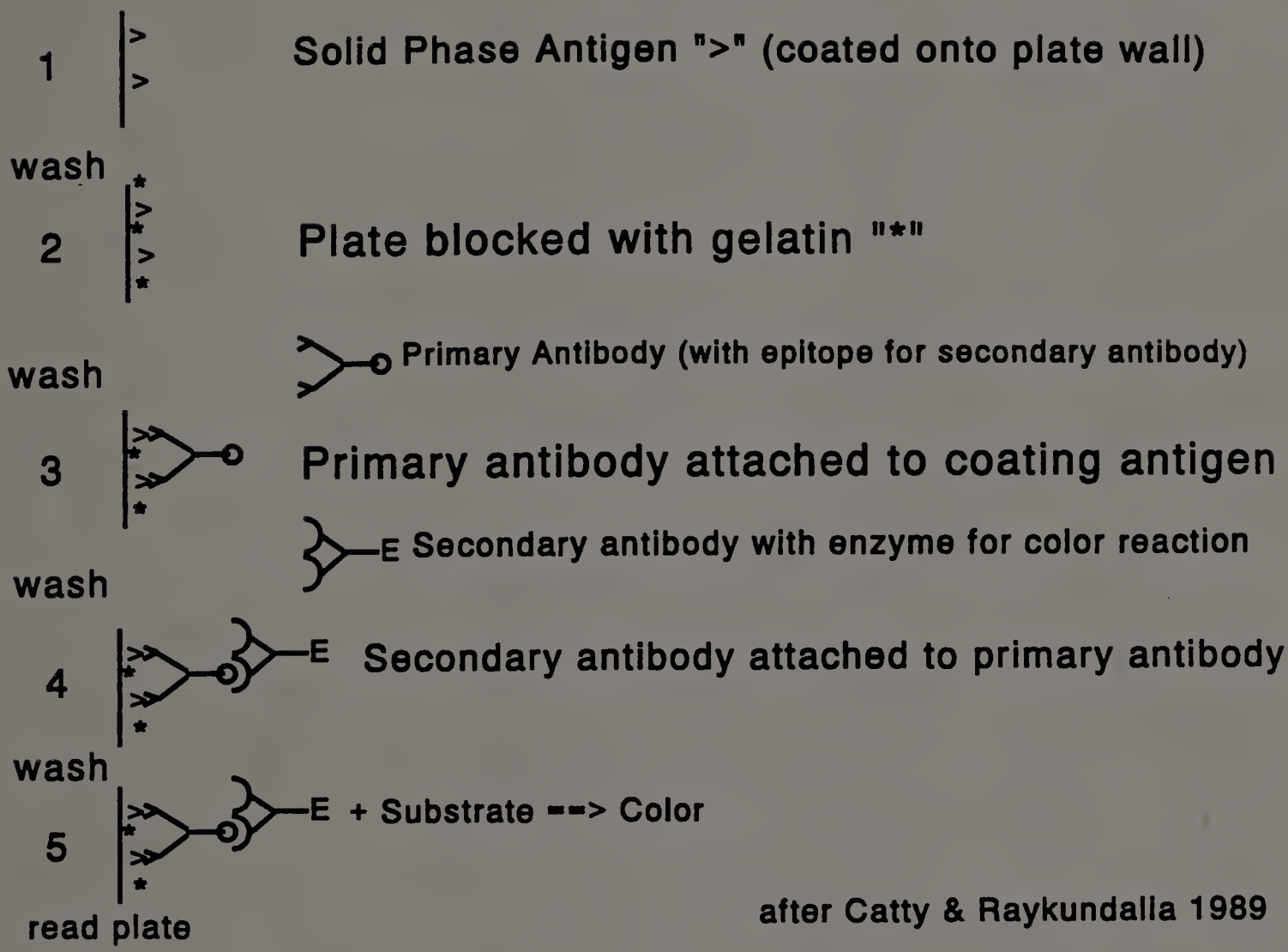


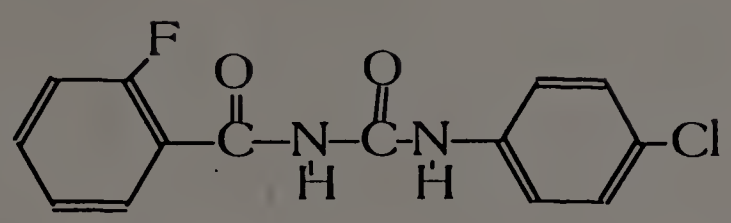
Fig. 44. Flow chart of procedure for indirect immunoassay of diflubenzuron.



after Catty & Raykundalia 1989

Fig. 45. Chemical structure of two inactive (i.e. not chitin systhesis inhibitors) analogues of diflubenzuron, from Nakagawa et al. 1992.

Inactive Diflubenzuron Analogues, from Nakagawa et al. 1992



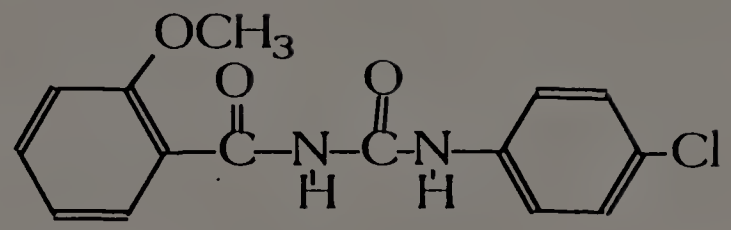


Table 5. Radioisotope ([^{3}H]-GlcN) incorporation by *Chironomus tentans*, 7 day incubation.

Diflubenzuron Concentration	**DPM (Standard Deviation, N[a])**	**DPM (Standard Deviation, N[a])**	**DPM**
(μ*M*)	Tube Incubation	Plate Incubation[b]	Published Results using Same Assay System[c]
0	389 (124.7, 8)	132 (58.6, 3)	10,000
5	401 (298.3, 6)	75 (77.9, 3)	3000
500	392 (211.1, 6)	ND	ND

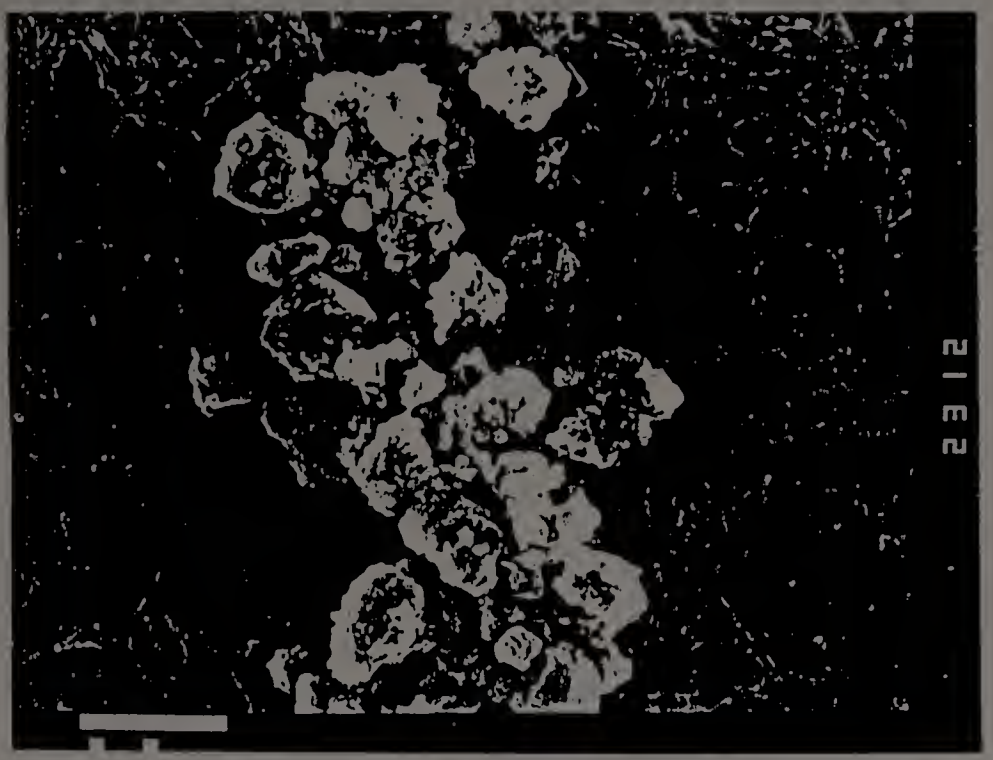
[a] N, number of replicates, does not reflect cases where radioisotope incorporated was not different from background counts.

[b] no significant inhibition by diflubenzuron; treatments not significantly different, t-test, $p>0.1$.

[c] Londershausen et al. 1988 using same chitin synthesis assay system of *C. tentans* cell culture; SIR 8514 (a diflubenzuron analogue) was inhibitor.

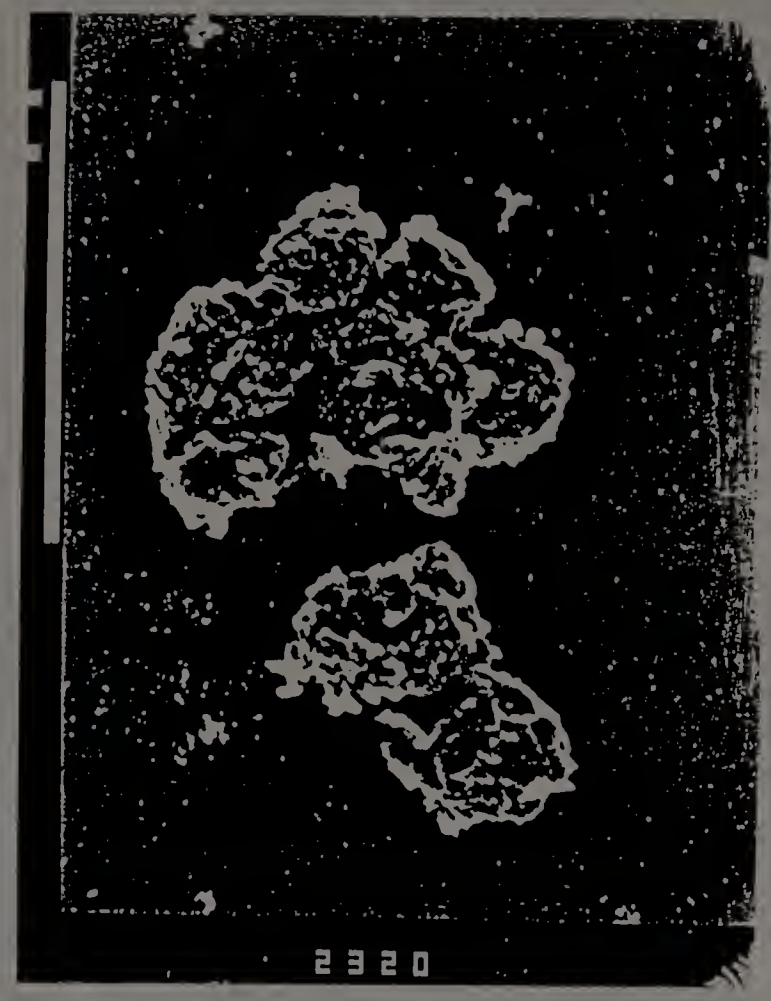
ND: not done

Fig. 46. SEM photographs of whole *Chironomus tentans* cells in extracellular matrix (A), whole cells without extracellular matrix after treatment with chitinase (B), and putative plasma membrane sheets with silica-polymer pellicle coat (C); bar = 10 μm.



A

B



C

Fig. 47. Standard curve for dolichol quantification on HPLC; dolichol homologues peak area summed; hexane/methanol/2-propanol/85% phosphoric acid (200:400:400:1, v/v) mobile phase at a flow rate of 1.1 ml/min through a 5 μm C_{18} Zorbax reverse phase column; UV detector set at 210 nm.

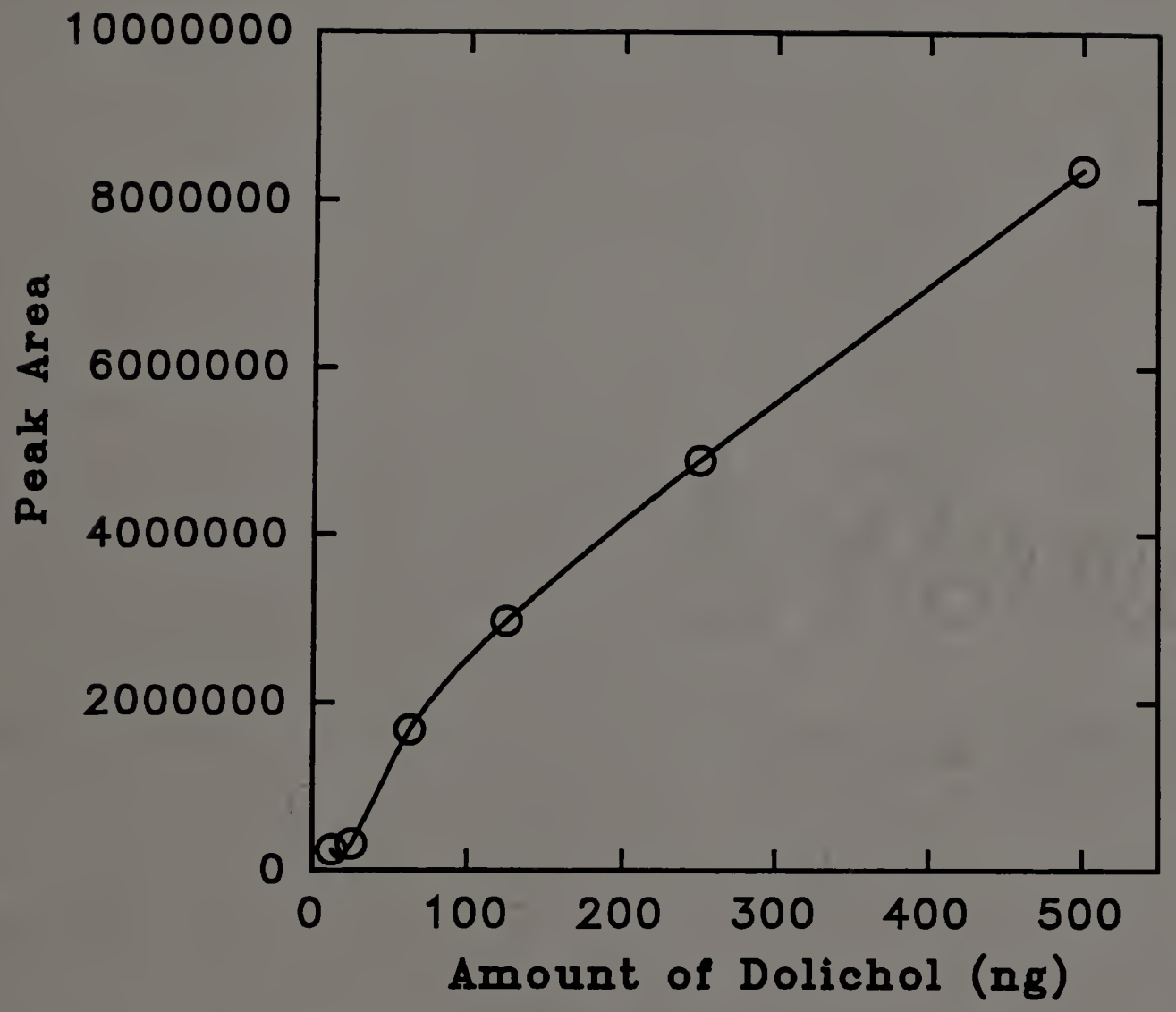

10000000
8000000
6000000
4000000
2000000
0
Peak Area
0
100
200
300
400
500
Amount of Dolichol (ng)

Fig. 48. HPLC chromatograph of the lipid extract of whole *Chironomus tentans* cells (200 µg); hexane/methanol/2-propanol/85% phosphoric acid (200:400:400:1, v/v) mobile phase at a flow rate of 1.1 ml/min through a 5 µm C_{18} Zorbax reverse phase column; UV detector set at 210 nm.

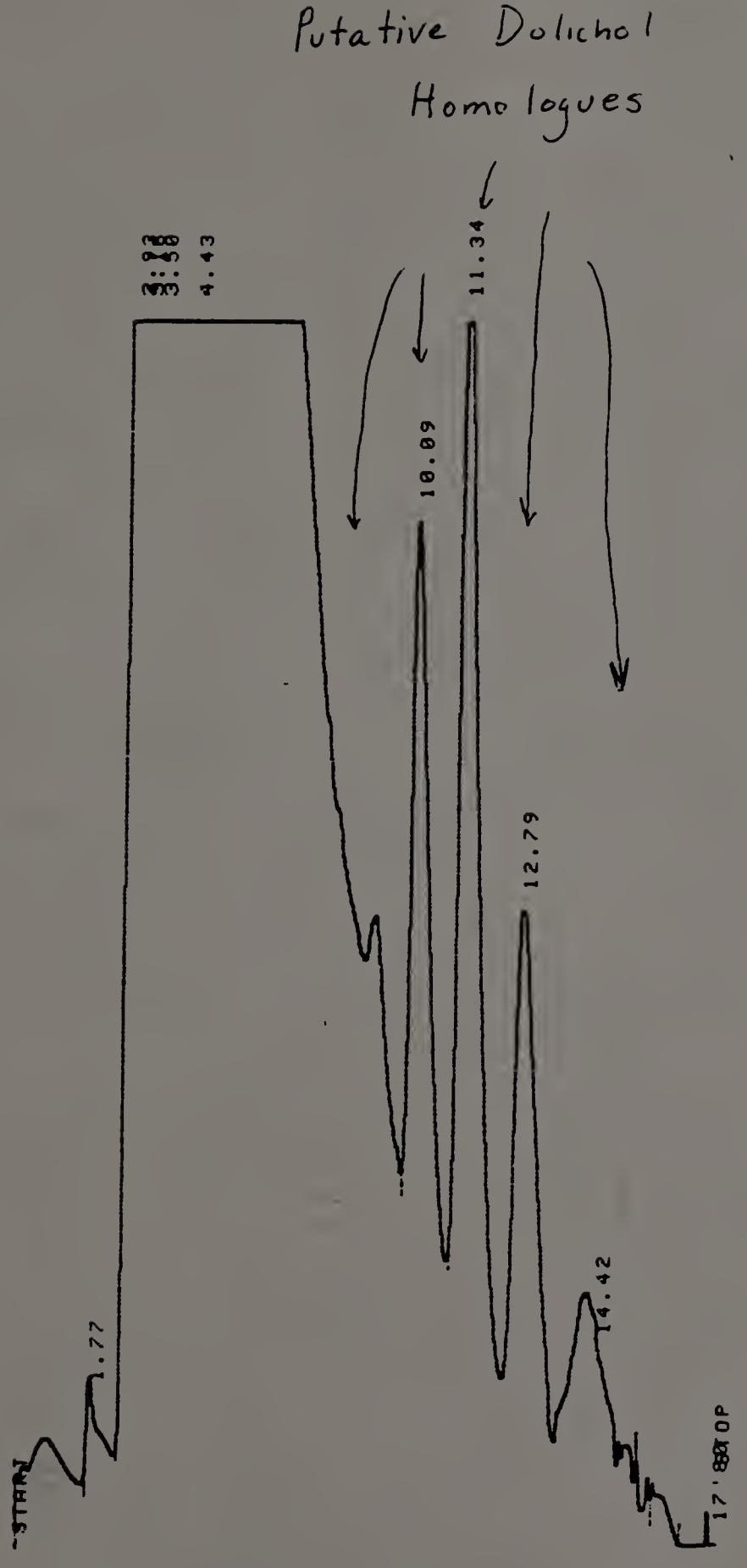

Putative Dolichol
Homologues
11.34
10.09
12.79
14.42
4.43
1.77
TIME (min)

Table 6. Thin layer chromatography of putative Dol-PP-GlcNAc.

Solvent	R_f		
	Smaller Volume Applied to Plate	Larger Volume Applied to Plate	Published Results[b]
A (chloroform/methanol/water, 60:35:6, v/v)	0.3	0.29[a] 0.20[a]	0.27 0.18
B (chloroform/methanol/acetic acid/water, 50:25:7:3, v/v)	0.79	ND	0.73
C (chloroform/methanol/ammonium hydroxide, 75:25:4 v/v)	0.05	ND	0.08

[a] 0.20 spot smaller than 0.29 spot.
[b] behavior of Dol-PP-GlcNAc on Merck silica plates, reported in Heifetz & Elbein 1979.
ND: not done

Fig. 49. HPLC chromatograph of dolichol; hexane/methanol/2-propanol/85% phosphoric acid (200:400:400:1, v/v) mobile phase at a flow rate of 1.1 ml/min through a 5 μm C_{18} Zorbax reverse phase column; UV detector set at 210 nm.

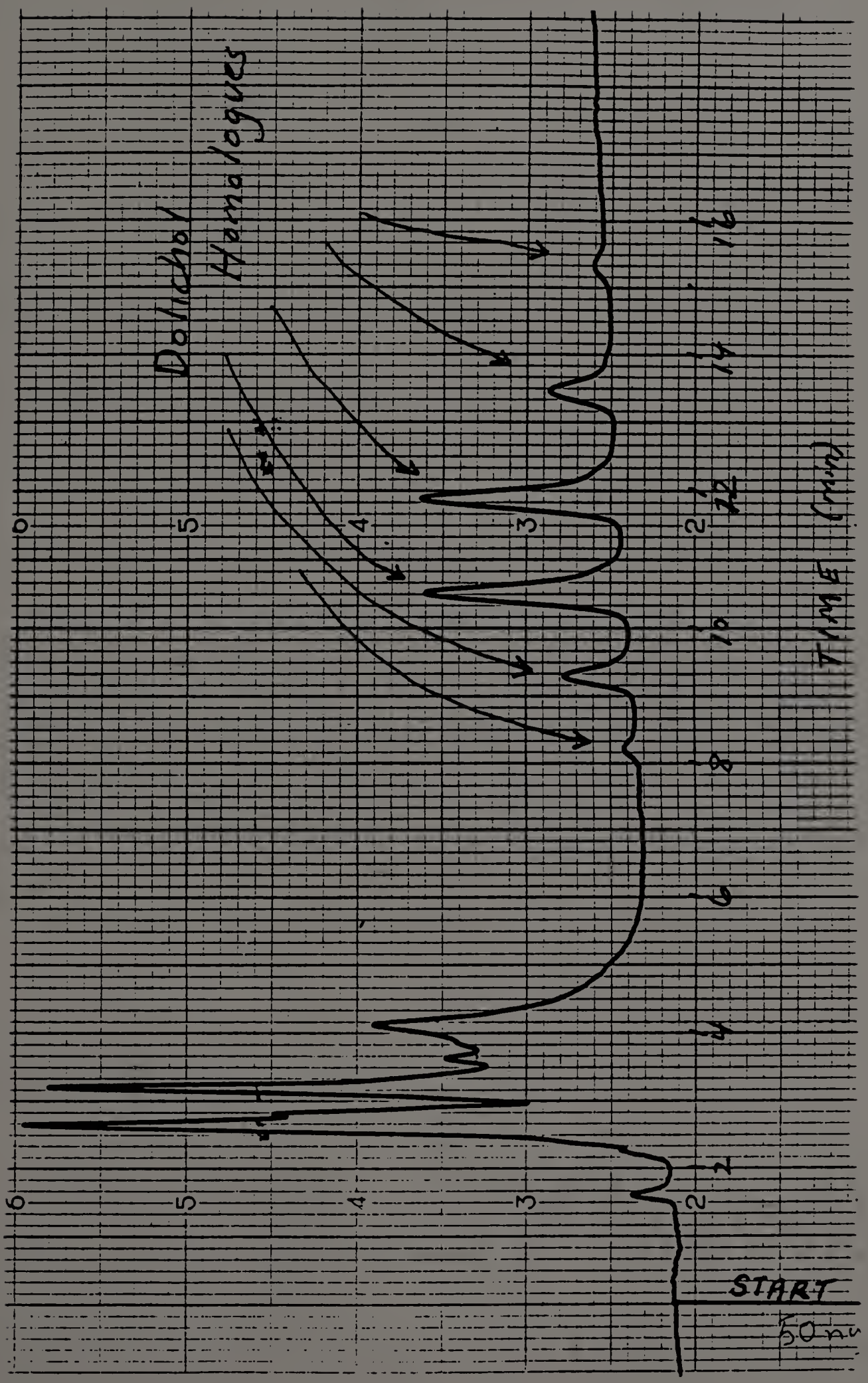
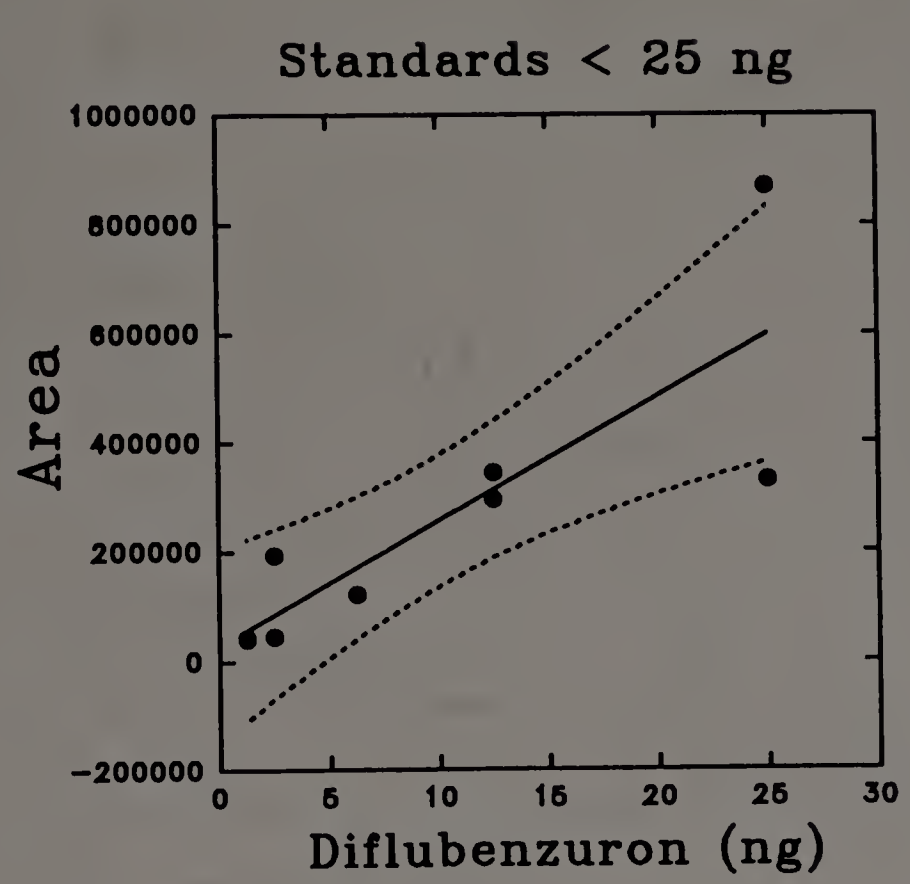

Dolichol Homologues
6
5
4
3
2
START
50 mv
Time (min)
14
16
18
8
10
12

Fig. 50. Standard curve for diflubenzuron quantification on HPLC; acetonitrile/water (60:40, v/v) mobile phase at a flow rate of 1.1 ml/min through a 5 μm C_{18} Zorbax reverse phase column; UV detector set at 254 nm; dashed lines are 95% confidence intervals.

Diflubenzuron Standards, HPLC



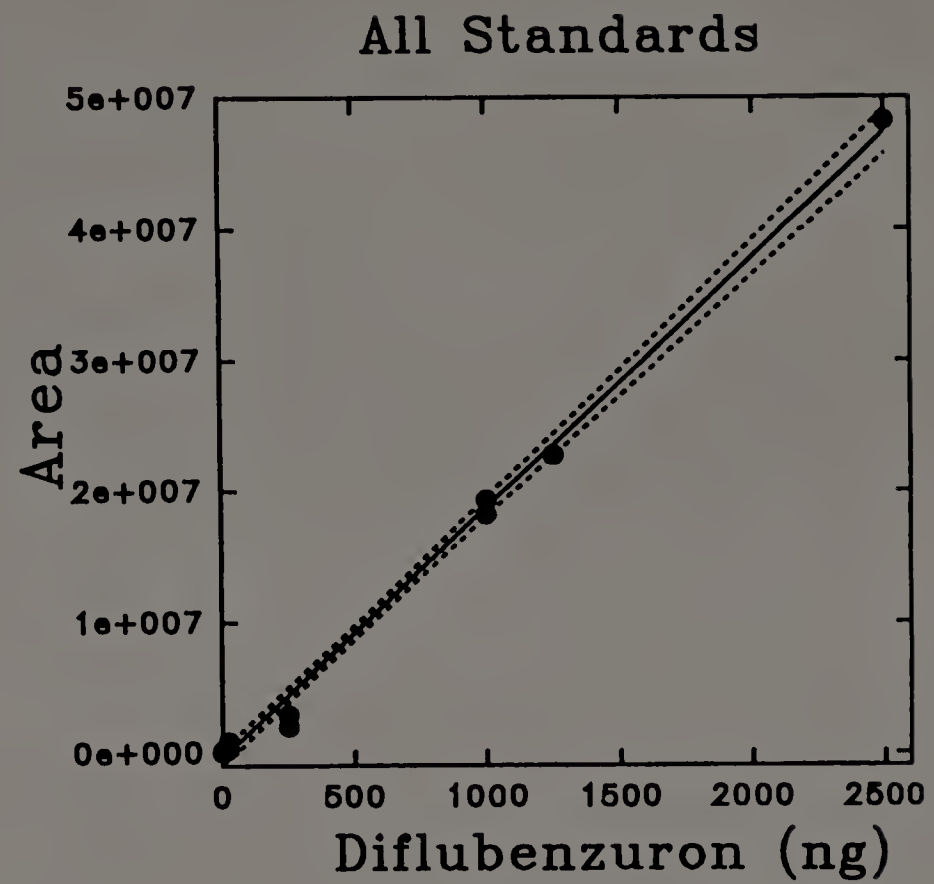


Fig. 51. Inhibition of binding of primary antibody (anti-diflubenzuron) by various haptens (diflubenzuron, inactive analogues) in an indirect immunoassay in which the secondary antibody (IgG) was conjugated with alkaline phosphatase; coating antigen (N-(Carboxypropyl)diflubenzuron--ovalbumin) was diluted 1:500, primary antibody was diluted 1:500 and pre-incubated with 1% BSA, secondary antibody was diluted 1:3000, and substrate (*p*-nitrophenyl phosphate) incubated for 35-40 min.

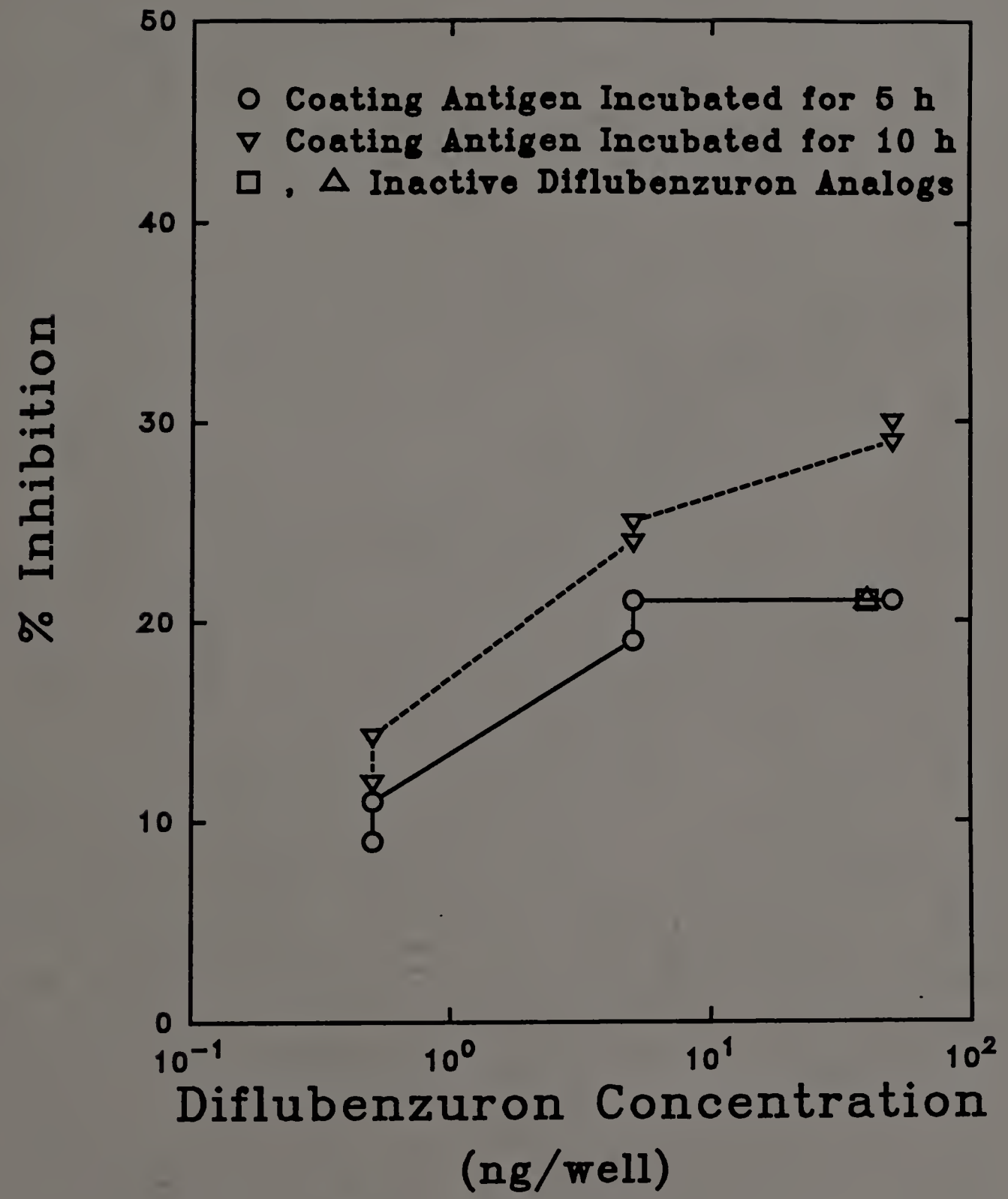
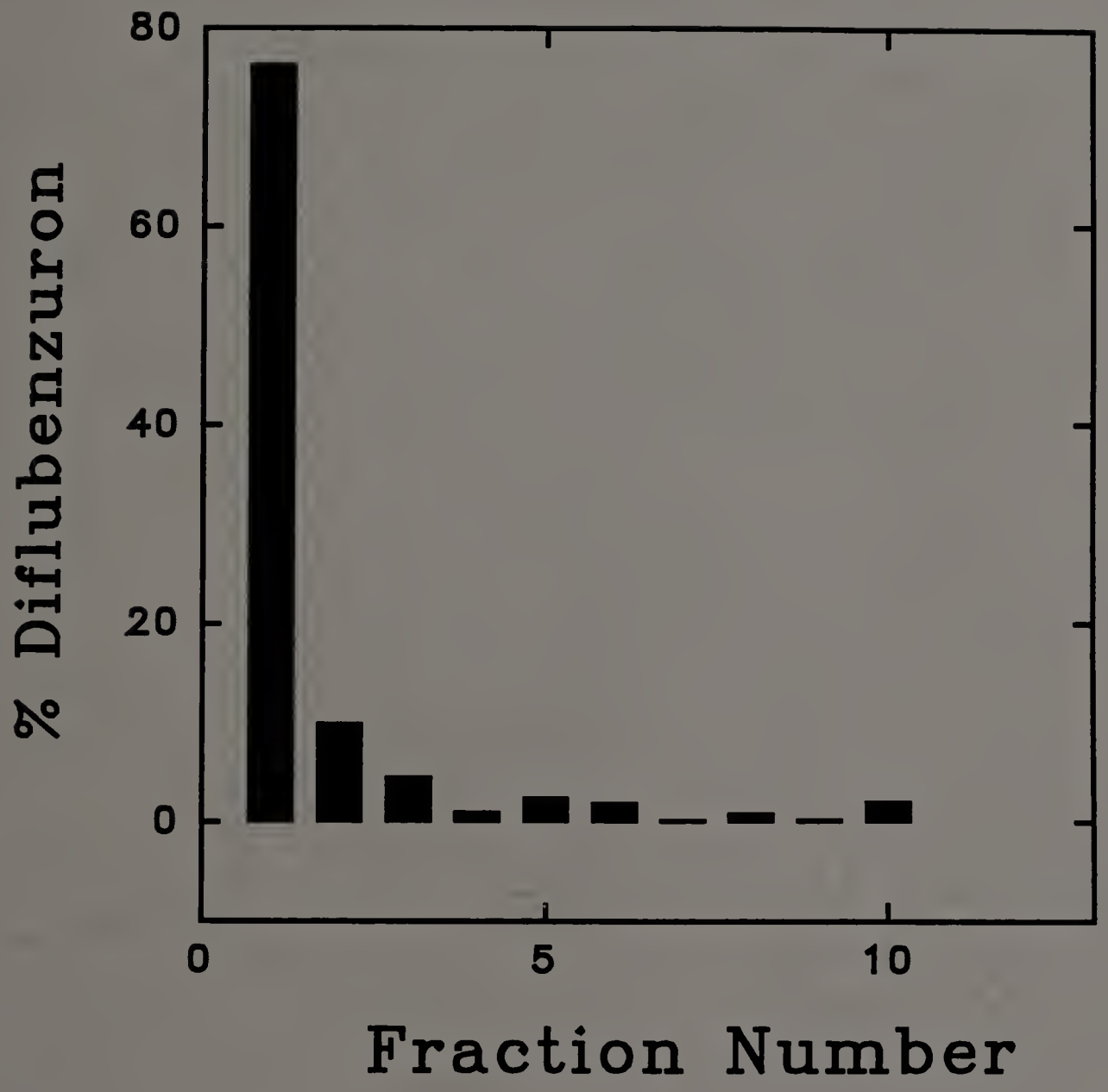

50
40
30
20
10
0
% Inhibition
○ Coating Antigen Incubated for 5 h
▽ Coating Antigen Incubated for 10 h
□ , △ Inactive Diflubenzuron Analogs
10^{-1}
10^{0}
10^{1}
10^{2}
Diflubenzuron Concentration
(ng/well)

Fig. 52. Diflubenzuron elution from a C_{18} solid support disk following 20 ml of acetonitrile/water (75:25, v/v); quantified by HPLC, acetonitrile/water (60:40, v/v) mobile phase at a flow rate of 1.1 ml/min through a 5 µm C_{18} Zorbax reverse phase column; UV detector set at 254 nm.


% Diflubenzuron
80
60
40
20
0
0
5
10
Fraction Number

APPENDIX A

ABBREVIATIONS

ATP: adenine triphosphate
BSA: bovine serum albumin
Dol: dolichol
Dol-P: dolichol monophosphate
Dol-PP-GlcNAc: dolichol pyrophosphate N-acetyl-D-glucosamine
Dol-PP-(GlcNAc)2: dolichol pyrophosphate N-acetyl-D-glucosamine N-acetyl-D-glucosamine
DTE: dithioerythritol
EGTA: ethylene glycol-bis-(β-aminoethyl ether)
GlcN: glucosamine
GlcNAc: N-acetyl-D-glucosamine
glygly: glycine-glycine buffer
MES: 2[N-morpholina]ethansulfonic acid
PBS: phosphate buffered saline
UDP-GlcNAc: uridine diphosphate N-acetyl-D-glucosamine

APPENDIX B

LIST OF STREAM TAXA

Stoneflies (Plecoptera)
- *Amphinemora**
- *Haploperla*
- *Leuctra* type A*
- *Leuctra* type B*
- *Ostrocerca**

Caddisflies (Trichoptera)
- *Aphropsyche*
- *Dolophilodes*
- *Hydatophylax*
- *Ironoquia**
- *Limnephilus*
- *Lepidostoma**
- *Marilia*
- *Molophilus*
- *Neophylax**
- *Polycentropus*
- *Pycnopsyche*
- *Rhyacophila**
- *Wormaldia*

True Flies (Diptera)
- Ceratopogonidae
- Chironomidae*
- *Chaoborus*
- *Dicranota*
- Dolochopodidae
- *Gonomyia*
- *Pedicia*
- *Phalacrocera*
- *Prosimulium magnum**
- *P. mixtum/fuscum* complex*
- *Simulium vernum* group*
- *S. vittatum**
- *Stegopterna mutata**
- *Tipulus*

Beetles (Coleoptera)
- *Agabus*
- *Hydroporus*
- *Optioservus*
- *Promoresia*

Mayflies (Ephemeroptera)
- Baetidae
- *Eurylophella*
- Heptageniidae
- *Siphlonurus**

Odonata
- *Aeshna*
- *Cordulegaster*
- Corduliidae/Libellulidae
- Gomphidae

True Bugs (Hemiptera)
- *Hesperocorixa*
- *Sigara*

Alderflies (Megaloptera)
- *Sialis*

Annelida

Acari

Amphipod

Cyclopoid Copepod

---

* common in both treated and control sites

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